Mycobacterium tuberculosis infection and disease

- a contribution to the understanding of immunological diagnostics in children.

PhD thesis

Synne Jenum, M.D.

Department of Pathology and Center for Immune Regulation
Institute of Clinical Medicine
Faculty of Medicine
University of Oslo

&

Department of Clinical Science
Faculty of Medicine and Dentistry
University of Bergen

Norway

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LIST OF PAPERS

I: **Influence of Age and Nutritional Status on the Performance of the Tuberculin Skin Test and QuantiFERON®-TB Gold In-Tube in Young Children Evaluated for Tuberculosis in Southern India**

Jenum S, Selvam S, Mahelai D, Jesuraj N, Cárdenas V, Kenneth J, Hesseling AC, Doherty TM, Vaz M, Grewal HMS*, TB Trials Study Group.

(The Pediatric Journal of Infectious Diseases, accepted April 2014, published ahead-of print 2nd May 2014)

II: **Identification of biomarkers for *Mycobacterium tuberculosis* infection and disease in BCG-vaccinated young children in Southern India.**

S. Dhanasekaran, Jenum S, Stavrum R, Ritz C, Faurholt-Jepsen D, Kenneth J, Vaz M, Grewal HMS*, Doherty TM, TB Trials Study Group.

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III: **Concordant or discordant results by the Tuberculin skin test and QuantiFERON-TB Gold In-Tube test in young children are reflected in biomarker profiles.**

S. Dhanasekaran[a], Jenum S, Stavrum R, Ritz C, Kenneth J, Vaz M, Doherty TM *, Grewal HMS, TB Trials Study Group.

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IV: **The frequencies of IFNγ+IL2+TNFα+ *Mycobacterium tuberculosis*-specific CD4+CD45RO+ T cells correlate with the magnitude of the Quantiferon Gold In-tube response in a prospective study of healthy Indian adolescents.**

Jenum S*, Grewal HMS, Hokey D, Kenneth J, Vaz M, Doherty M, Jahnsen FL, TB Trials Study Group.

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*Corresponding author

LIST OF ABBREVIATIONS

ACS	Adolescent Cohort Study conducted by the TB Trials Study group
AIDS	Aquired Immune Deficiency Syndrome
AP view	Anterior-posterior view (in the context of CXR)
APC	Antigen-Presenting Cell
BAL	Broncho-Alveolar Lavage by inhalation of hypertonic saline
BAZ	Body Mass Index-for-age Z-score
CDX	CD antigen number X
CFP-10	Culture Filtrate Protein 10
CM T cell	Central memory T cell
CPT	Cell Preparation Tube
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CVW	Case Verification Ward at Emmaus Swiss Hospital, Palamaner, India
CXR	Chest X-ray
DAG	Directed Acyclic Graph
DC	Dendritic Cell
DOTS	Directly Observed Treatment Short-course
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immuno-spot
EM T cell	Effector memory T cell
ESAT-6	Early Secretory Antigenic Target, 6 kDa
FBS	Fetal Bovine Serum
FDR	False discovery rate. Statistical method to adjust for multiple testing
FTT	Failure to thrive (defined in section 3.2.1.3)
GCP	Good Clinical Practice
GM-CSF	Granulocyte-macrophage colony-stimulating factor
γδ T	Gamma-delta T cells
HAZ	Height-for-age Z-score
HIV	Human Immune-deficiency Virus
IFNγ	Interferon gamma
IGRA	Interferon-gamma-release-assay (QuantiFERON Gold In-tube and T-SPOT)
IL-X	Interleukin number X
IP-10	IFNγ-inducible protein 10, same as CXCL10 (C-X-C motif chemokine 10)
IPT	Isoniazid Preventive Therapy (6-) 9 months
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LMIC	Low- to middle income country
LTBI	Latent Tuberculosis Infection
MCP-1	monocyte chemotactic protein-1, same as CCL2 (Chemokine (C-C motif) ligand 2)
MDGs	Millennium Developmental Goals
MDR	Multi-Drug-Resistant (MTB resistant to at least isoniazid and rifampine)

MMP	Matrix Metalloproteinases
MTB	*Mycobacterium tuberculosis*
NCS	Neonatal Cohort Study conducted by the TB Trials Study group
NHP	Non-human primates (monkeys)
NKT	Natural Killer T cells
NO	Nitric oxide
NTM	Non-tuberculous mycobacteria
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PD-1	Programmed Death-1
PPD	Purified Protein Derivate from *M.bovis*
PPR	Pattern recognition receptor
QFT	QuantiFERON Gold In-tube. A commercial IGRA produced by Cellestis
RAB33A	The RAB33A is a gene belonging to the RAS oncogene familiy
RORγt	RAR-related orphan receptor gamma, isoform t
RT	Room temperature
SEB	Staphococcal Enterotoxin B
SES	Socioeconomic status
SSI	Statens Serum Institute, Copenhagen, Denmark
STAT-3	Signal Transducer and Activator of Transcription 3
TB	Tuberculosis
TCR	T cell receptor
T_{CM}	Central Memory T cell
T_{EM}	Effector Memory T cell
TGF-β	Transforming growth factor beta
Th1	CD4+ T helper cell, type 1
Th17	CD4+ T helper cell, type 17
TNFα	Tumor Necrosis Factor alpha
T_{TE}	Terminally differentiated Effector T cell
Treg	Regulatory T cell (natural occurring or inducible)
TST	Tuberculin skin test/Mantoux
UN	United Nations
VEGF	Vascular Epithelial Growth Factor
WAZ	Weight-for-age Z-score
WHO	World Health Organization
WHZ	Weight-for-height Z-score
XDR	Extensively Drug-Resistant (MDR MTB also resistant to all fluoroquinolones plus kanamycin, amikacin or capreomycin)

1. INTRODUCTION

1.1. Tuberculosis

1.1.1. *Mycobacterium tuberculosis*

Robert Koch discovered in 1882 that tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (MTB). MTB is a highly aerobic, non-motile, non-encapsulated, non-spore-forming slender rod measuring 0.4 x 3 μm. The division rate is slow (15-20 hours). A peptidoglycan wall rich in lipidoglycans makes MTB extremely resistant to desiccation as long as it is protected from UV-light,[6] and constitutes an important survival strategy reflected in the high proportion of the bacterial genome (~30%) which is involved in the synthesis or metabolism of lipids.[7] MTB is not visible after gram staining but can be stained by acid-fast staining and is thus visible by Ziehl-Neelsen or auramine staining methods. MTB is one of 8 mycobacterial species within the MTB complex, which members are causative agents of human and animal tuberculosis. Other species capable of causing human tuberculosis are *M. bovis, M. bovis BCG, M. africanum and M. canetti,* but MTB is indeed the most important.[6]

1.1.2. Infectious spread and clinical presentation

Pulmonary TB is the most frequent clinical presentation that accounts for 60-90% of the TB cases depending on ethnic background, age, co-morbidity and immune status as well as the genotype of the MTB strain.[8] The classical symptoms of pulmonary TB are chronic purulent cough, loss of appetite and weight, fever and night sweats and hemoptysis,[9] but these symptoms might be less prominent in children <3 years and in patients with HIV infection or other immunosuppressive diseases.[10-12] Subjects with pulmonary TB constitute the infectious source by shedding bacteria while coughing,[13, 14] and such a patient infects on average 10-15 persons each year if left untreated.[15] Close household contacts of the index case have the highest exposure to infected aerosols and are very likely to get infected[16] as <10 bacilli might be sufficient to establish an infection in the lung.[6] Corresponding to an evolving delayed-type hypersensitivity reaction towards mycobacteria, a primary complex, or Gohn focus which consists of a granuloma, usually located close to the pleura in the lower part of

the upper lobe or in the upper part of the lower lobe, and enlarged adjacent hilar lymph nodes, provides radiologic evidence of an established lung infection.[17]

About 5-10% of infected subjects develop TB disease during their lifetime,[15] half within the first 18-24 months of infection.[18, 19] Without re-exposure the risk gradually declines.[20, 21] Bacterial growth is favored in the upper lung-lobes rich in oxygen,[4] explaining the typical localization of TB lesions in adult cavitary disease[22]. Extrapulmonary disease occur in 15-30% of cases and can be localized to virtually any organ causing lymphadenitis (30%), TB of the urogenital tract (16-20%), osteoarticular TB (7-15%), meningitis or tuberculoma (4%) or pericarditis (0.14-0.3%).[23] Children <2 years at the time of MTB infection and immune compromised subjects are at increased risk of progression to TB and more susceptible to severe manifestations like disseminated TB and meningitis[22, 24-27] (Table 1).

Disease manifestation	<1 year at infection	1-2 years at infection	>10 years at infection
No disease	50%	70-80%	98%
Pulmonary disease (3-7 months postinfection)	30-40% (Ghon focus, lymph node or bronchial)	10-20% (Ghon focus, lymph node or bronchial)	30-40% (effusion or adult type)
TB meningitis or miliary (1-3 months postinfection)	10-20%	2-5%	<0.5%

*adapted from Marais et al. State of the Art. Int J Tuberc lung dis, 2004, vol 8 [22].

Table 1. The manifestations of TB depend on age at infection

More than 90% of immune competent adults will naturally control the infection and develop no symptoms.[28] The current paradigm states that few will eradicate the bacteria,[5, 14, 29] however, some studies raise the possibility that some persons eliminate MTB shortly after infection[30] or even later if protected from re-exposure.[20] Evidence of previous MTB exposure and latent tuberculosis infection (LTBI) can be provided by the presence of a primary complex/Gohn focus on chest X-ray, and/or a positive tuberculin skin test (TST/Mantoux) or interferon-gamma-release-assay (IGRA).[12] Nevertheless, MTB persistence represents a risk of TB progression,[31]

particularly if the immunity wanes as may be seen by HIV co-infection or by administration of immune-suppressive drugs.[32, 33] The life-time risk of TB in HIV co-infected subjects is estimated to 30-40%.[15]

1.1.3. History of the tuberculosis epidemic

MTB and man have co-existed since their common origin in Africa.[34] Six phylogenetic MTB lineages (lineages which share the same ancestor) exist which associate with 6 different geographic regions on the globe, and thus constitutes a sympatric pattern of host-pathogen co-existence (Sympatric: host and pathogen originates from the same geographical region).[34, 35] Transmission of MTB is facilitated in sympatric host-pathogen combinations compared to allopatric host-pathogen combinations (host and pathogen originates from different geographical regions).[35] The earliest evidences of TB in man are provided by lesions in fossil bones that date back to about 8000 BC. TB, historically named phthisis or consumption, has been a constant threat to mankind, hitting hardest in densely populated urban settings.[36] TB was the leading cause of death in the western world in the 19th century, responsible for up to 20 % of deaths.[37] The TB prevalence started to decline as a result of general public-health initiatives like isolation of patients in institutions, and not as a consequence of vaccination and treatment.[37, 38] This can be exemplified by London, where the decline started after implementation of the Poor Law (1834) which stated that relief for the poor should only be given within the walls of the workhouses. After Koch's discovery of the tubercle bacillus (1882) and the emerging assumption of infectivity,[39] Prussian physicians introduced strict isolation and treatment of patients in sanatoriums.[36] This led to a more rapid decline in TB prevalence in Prussia compared to other European countries and the United States, despite the fact that Prussia had the most crowded living conditions at that time.[37] In 1921, a vaccine, Bacille Calmette-Guérin (BCG), developed by Calmette and Guérin was introduced, and proved to protect children,[36] but could not prevent pulmonary disease in adults.[40] Furthermore, the sanatorium treatment by the strict rest cure consisting of "no known consumptions" (bed rest, fresh air, sun, healthy foods), optimized the patients capacity to fight MTB and supplemented the collapse therapy (1930s) (pneumothorax and thoracoplasty).[36] Nevertheless, the mortality remained high until the discovery of the effect of streptomycin on TB (Waksman 1943). Other anti-TB agents were introduced shortly thereafter,[36, 41] and Sir John Crofton initiated extensive trials of multi-drug

regimens in order to prevent relapses by resistance to drugs used in mono-therapy. In the early 1970s, the introduction of rifampicin enabled entirely oral administrations for 6 months (short course) compared to the 18-24 months previously required for cure. With the introduction of successful treatment, long term sanatorium isolation was no longer needed and TB gradually got under control in the western world. Subsequently, the scientific and political interest in TB declined and TB surveillance was neglected. The burden of TB in the developing world was never addressed.[38, 42-44] In the late 1980s, the emergence of the HIV/AIDS epidemic and multi-drug-resistant (MDR) and extensively drug-resistant (XDR) MTB strains became an increasing threat to the western world. This proved the necessity for global initiatives to fight the TB epidemic. In 1993, WHO declared TB a global emergency.[45]

1.1.4. "Cure is the best prevention" (WHO)

The World Health Organization (WHO) first focused on a global commitment to cut transmission by early detection of contagious pulmonary TB cases and effective treatment through the framework of Directly Observed Treatment Short-course (DOTS).[46] DOTS includes governmental commitment to 1) active detection of TB cases, 2) standardized treatment directly observed by a health worker for at least 2 months, 3) drug supply and 4) a standardized recording and reporting system that allowed assessment of treatment results. Despite successful implementation of DOTS programmes worldwide, the impact on TB incidence have been much less than estimated. This is likely attributable to changes in demographic-, epidemiological- and social factors which independently or by interaction increase the transmission and vulnerability to TB in the population.[43, 44, 47] In 2000, United Nations (UN) Member States (191) agreed upon 8 Millennium Developmental Goals (MDGs) "for a better world".[48] MDG 6, Target C, concerns TB, and states that TB incidence should be halted and begin to reverse within 2015. The Stop TB Partnership was established to develop a strategy to achieve this goal, but ultimately defined more ambitious goals: 1) by 2015, half the prevalence and death rates from the 1990 baseline, and 2) by 2050, eliminate TB as a public health problem (defined as an incidence $\leq$ 1 TB case per 100 000). The consensus was that full implementation of The Global Plan To Stop TB 2006-2015 (Stop TB Strategy) would be needed for these targets to be met.[49] While simultaneously aiming at addressing social determinants for TB, expansion of the DOTS Strategy is still the fundamental basis of the Stop TB Strategy, but the

ultimate goal of elimination of TB cannot be achieved unless new tools (diagnostics, drugs and vaccines) are made available.[43, 50] Therefore, promotion, support and coordination of relevant research are key elements in the Stop TB Strategy.[49]

1.1.5. The burden of tuberculosis today

Today, the global TB burden is considerable with an estimated 9.0 million new cases and 1.5 million deaths in 2013. HIV co-infection was present in 13% of new cases and HIV-patients accounted for 360 000 deaths. India had the largest number of incident cases in the world (2.0-2.3 million), which accounted for 26% of the global cases. The incidence rate fell at an average of 1.5% per year in the periode between 2000 and 2013, ensuring that the MDG Target 6.c of halting and reversing the global TB incidence by 2015 has been achieved. Notably, an accelerated decline is required to achieve the goals of halving TB mortality and prevalence rates, but this is. Of the 22 high burden countries that accounted for 82% of the world's TB cases, 10 appear on track to meet all 2015 targets for reductions in incidence, prevalence and mortality.[51] The risk of TB associates with low socio-economic status, but are affected by migration, increased urbanization and changes in demographics and life-style. The ongoing financial crisis already hinders tuberculosis-control programmes,[28] and might therefore change the estimates of the TB burden by 2015 alone, or together with other political- and/or climate changes.[44, 47] Furthermore, the promising gains in TB control are threatened by MDR- and XDR-TB, which constitutes 3.5% of new cases.[51]

Latent TB infection is widespread and an estimated 2 billion individuals are latently infected around the globe.[52] These subjects constitute a huge reservoir for TB disease and transmission,[15] but the proportion of exposed/infected subjects who clear the infection is unknown.[53] Pre-existing immunity in subjects with latent infection (or as a result of BCG vaccination) is likely to mediate accelerated containment of MTB in the case of re-infection, but does not provide full protection and might even cause a more violent tissue-destroying hypersensitivity reaction.[53] Transition from latent TB to active TB is facilitated by factors which impair the host immunity. In 22 high burden countries which together have 80% of the estimated total TB burden, Lönnroth and Raviglione estimated the proportion of TB cases that could be attributed to specific risk factors (population attributable fraction (PAF)): malnutrition; 34%, smoking; 23%, HIV; 7%, diabetes; 6%, harmful alcohol use; 13% and indoor air

pollution; 26%. The relative importance of these factors depends on their prevalence, which differ between countries and regions.[43] In addition, genetic variety in MTB strains affects virulence and transmission, and genetic variations between populations and individuals affect the susceptibility to TB.[8, 44]

1.1.6. The diagnosis of MTB infection and disease

The epidemiological situation and health resources differ considerably around the globe. In order to optimize the management of TB in the local setting, WHO has recommended the elaboration of national- or regional guidelines for diagnosis of MTB infection (latent TB) and disease (active TB).[54-57]

1.1.6.1. MTB infection (latent TB)

The diagnosis of MTB infection is relevant in assessing risk of TB in recently exposed contacts of infective TB cases, particularly in children aged <5 years,[58] in screening of immigrants from TB high-endemic countries, HIV patients, and patients selected for immunosuppressive therapy. MTB infected subjects at risk of developing TB should generally be considered for isoniazid preventive therapy (IPT).[58-60] The diagnosis relies on immunological tests (TST and/or IGRAs) which detect prior sensitization with mycobacterial antigens. The validation of both tests is complicated by the lack of a gold standard for latent MTB infection. Their performance depend on the population at risk and both tests have shortcomings in terms of sensitivity and specificity.[61-64] Notably, Both TST and IGRAs are unable to conclude on 1) whether live MTB bacilli are present – a strict requirement for reactivation, and 2) the time-point of infection, an important risk factor for TB progression.[53] The ability of the tests to predict TB progression largely depends on the context: in low incidence settings the positive and negative predictive value of IGRAs seems better than TST, and the negative predictive value of IGRAs in this setting is very good. Notably, these findings are not valid in high incidence settings, making TST and IGRAs inappropriate tools to rule out TB.[65-67] Importantly, the negative predictive value of IGRAs remains to be established in children and immunocompromised individuals.[67]

1.1.6.1.1. Tuberculin Skin Test (TST)

TST is an ancient tool which measures the *in vivo* delayed type hypersensitvity (DTH) in response to intradermally injected purified protein derivate (PPD) from an

attenuated *M. bovis* strain. The standard TST consists of 0.1ml with 2 tubercular units (TU) of Statens Serum Institute (SSI) tuberculin RT23. Some countries/regions use, PPD manufactured from other laboratories resulting in some variation in the *M. bovis* strain used. The test is read by recording the degree of induration measured in mm 48-72 hours later. The induration results from a local inflammation triggered by memory T cells recognizing mycobacterial antigens presented by antigen-presenting cells (APCs) at the injection site (see 1.2 for further elaboration). TST conversion occurs about 6 weeks after exposure.[68] The cut-off for a positive test depends on the epidemiological setting (mycobacterial exposure), BCG-vaccination and host immunity (Table 2).

Table 2. The interpretation of TST depend on risk of exposure and clinical factors

Induration of ≥ 5 mm is considered positive in	**Induration of ≥ 10 mm is considered positive in**	**Induration of ≥ 15 mm is considered positive in**
● Human immunodeficiency virus (HIV)-positive persons. ● Recent contacts of TB patients. ● Persons with fibrotic changes on chest radiograph consistent with prior TB. ● Patients with organ transplants and other immunosuppressed patients (receiving the equivalent of 15 mg/d of prednisone for 1 month or more. Risk of TB in patients with corticosteroids increases with higher dose and longer duration.)	● Recent immigrants (i.e., within the last 5 years) from high-prevalence countries. ● Injection drug users. ● Residents and employees† of the following high-risk congregate settings: prisons, nursing homes and other long-term facilities for the elderly, hospitals and other health care facilities, residential facilities for patients with acquired immunodeficiency syndrome (AIDS), and homeless shelters. ● Mycobacteriology laboratory personnel. ● Persons with the following clinical conditions indicating higher risk: silicosis, diabetes mellitus, chronic renal failure, some hematologic disorders (e.g., leukemias and lymphomas), other specific malignancies (e.g., carcinoma of the head, neck, or lung),weight loss of 10% of ideal body weight, gastrectomy, and jejunoileal bypass. ● Children < 4 years of age, or infants, children and adolescents exposed to adults at high-risk	● Persons with no known risk

Center for Disease Control and Prevention, 2013. [69]

PPD contains antigens widely expressed by mycobacteria. Subsequently, BCG-vaccination after infancy and exposure to NTMs might affect the TST response, resulting in loss of specificity.[65] As TST is an *in vivo* test has been concern of a boosting response by repeated testing.[54]

1.1.6.1.2. Interferon-gamma-release assay (IGRA)

IGRAs measure the IFNγ-release after *in vitro* stimulation (16-24 h) of whole blood with the antigens ESAT-6 (6 kDa Early Secretory Antigenic Target), CFP-10 (Culture Filtrate Protein 10) expressed by the region of difference (RD-1 region) of the mycobacterial genome and secreted by MTB during active replication.[70] The RD-1 region is present in a few mycobacterial species only (*M. tuberculosis, M. africanum, M. kansasii, M. marinum leprae, M. marinum, M. smegmatis and M. szulgai*)[71] which increases the test specificity compared to TST both with regard to prior BCG-vaccination and NTM exposure.[65] Two IGRAs are currently commercially available:

- QuantiFERON Gold In-tube (QFT), Cellestis, includes the antigens ESAT-6, CFP-10 and TB7.7 (RD-11 antigen[53]) and measures the amount of IFNγ released from whole blood (ELISA platform).[72]
- T-SPOT.*TB*, Oxford Immunotec, includes the antigens ESAT-6 and CFP-10 and measures the number of effector T-cells that respond with IFNγ-production (by ELISPOT). The individual PBMC count is corrected for.[73]

1.1.6.2. Tuberculosis (active TB)

When active TB is clinically suspected at least two early morning (fasting) specimen should be obtained from sputum, gastric lavage or adequate tissue aspirates (ex: lymphadenitis). Specimens are stained with Ziehl-Neelsen (light microscopy) or auramine (fluorescence microscopy) to enable detection of acid-fast bacilli by direct microscopy. In addition, all specimens should be cultured for confirmed identification of MTB (designated confirmed TB) when adequate facilities and resources are available.[74] Liquid culture systems are recommended, but solid culture medium (Lövenstein-Jensen) is more cost-effective in resource-limited settings. Drug-susceptibility testing is recommended.[52] Adequate specimens can be difficult to obtain in extrapulmonary- and paucibacillary disease which are more frequent in the immunocompromised patient[74] and in children <5 years[12]. As a consequence, the diagnosis remains unconfirmed in 70-80% of young children (designated probable TB).[75-77] A new molecular diagnostic test (Xpert MTB/RIF assay) detects the MTB complex (defined in section 1.1.1) and rifampicin resistance (proxy for MDR) within 2 hours but is more resource intensive and less sensitive than liquid culture.[52, 75]

Therefore, in clinical settings involving children <5 years and/or immune-compromised patients the diagnosis of TB also relies on:[58]

- a history of known TB exposure (contact with an infectious index case).
- symptoms[10]
- radiologic lesions consistent with TB (chest, spine/bone or brain).

TST or IGRAs might be tempting to use as evidence of a previous encounter with MTB, but neither test can be used as a rule-out test, nor distinguish latent from active TB, and are therefore not recommended in the diagnosis of active TB.[54, 56, 57, 74, 78] Nevertheless, in smear-negative symptomatic children with a chest X-ray consistent with TB, either known exposure or a positive TST/IGRA was until recently recommended for the diagnosis of active TB.[74] Reduced sensitivity of TST/IGRAs in young children can therefore result in under-diagnosis of TB. New recommendations for the diagnostic approach in young children include a broader clinical evaluation.[12]

1.2. The host immune response to *Mycobacterium tuberculosis* infection and host-pathogen interaction

The outcome of MTB infection; immediate progression to TB, latent infection (with or without later re-activation resulting in TB) or clearance (with or without induction of adaptive immune responses) depends on the host-pathogen interaction.[1, 79] The host response is also influenced by BCG-vaccination or exposure to other mycobacteria,[80] age (young children[81] or elderly[82]), genetic factors,[83, 84] malnutrition[85] and vitamin deficiencies (vitamin A[86], vitamin D[87, 88], co-infections (HIV[15] and helminthes[89, 90]), immune-modulating diseases (diabetes[91, 92]), behavior (indoor pollution, smoking,[92, 93] alcoholism[94]) and drugs (TNFα-antagonists[33]).

1.2.1. Some basic concepts in immunology

Before proceeding further, some basic concepts often referred to in immunology are introduced: 1) Innate versus adaptive immunity and 2) Primary versus secondary immune responses. The innate immune system consists of epithelial cells, immune cells residing in the particular tissue (e.g. macrophages, dendrittic cells (DCs), mast cells) or recruited from blood (neutrophils, monocytes, NK-cells), and provides a rapid first line of defense against pathogens which probably eradicates many

infections without the need for deploying the adaptive immune system. Activation of the complement cascade further potentiates the efficiency of innate immune cells. When the innate immune system is overwhelmed by a new pathogen, a primary immune response develops. A primary immune response is characterized by innate responses which aim at keeping the pathogen sufficiently under control while more efficient adaptive immune responses by specific T- and B-cells evolve and enable pathogen clearance or dormancy. Later exposure to the same pathogen either by re-infection or re-activation activates immunological memory exhibited by memory T- and B-cells and enables a more rapid deployment of adaptive immune responses, characteristic of a secondary immune response.[95]

1.2.2. The immune system of the lung – an overview in the context of MTB infection

MTB's site of entry is the lung alveoli, thus herein, the focus is on immunological mechanisms within the lung parenchyma and lymph nodes draining this tissue. The mucosal surfaces of the respiratory tract are constantly exposed to inhaled antigens. Efficient immune responses which are vital in the context of invading pathogens might be devastating if induced by ubiquitous harmless antigens. Therefore, the immunological homeostasis in the human lung is biased towards an anti-inflammatory or regulatory phenotype in the steady-state. [96] While not the scope of this thesis, this aspect is important to keep in mind during the ensuing discussion of the host immune response to MTB infection.

1.2.2.1. The first pathogen encounter activates innate host immune responses

Whereas the conducting airways are protected against invaders by ciliated bronchial epithelium and secretory goblet cells which together enables mucociliary clearance of inhaled antigens, this robust barrier is lacking in the alveoli; MTB's main port of entry. Both in conductive airways and in the alveoli, macrophages and DCs are strategically positioned close to the epithelial surface to serve their functions of antigen capture and pathogen killing by phagocytosis (macrophages)[96] (Figure 1).

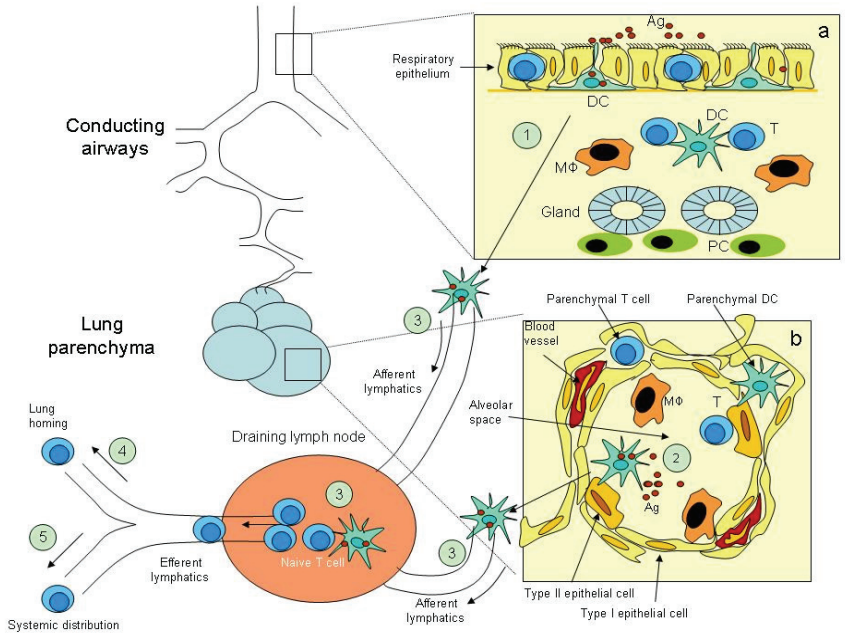

Figure 1. Initiation of a primary immune responses in the conductive airways (1) and in the lung alveoli (2). Panel a) and b) illustrate the location of immune cells and antigen (Ag) in 1 and 2 respectively. Resident DCs activated by Ag recognition travel through the afferent lymphatics to the draining lymph nodes where naive Ag-specific T-cells are activated to differentiate and proliferate (3) before they enter the circulation by the efferent lymphatics..Effector T cells subsequently home to the infected airways (4) whereas memory T cells are systemically distributed (5) to assure renewal of the effector T cell pool and immunological memory. Adapted from Frode L. Jahnsen.

Macrophages and DCs express a large range of pattern recognition receptors (PRRs) on their surface that recognizes different components of microorganisms. Some PRRs that recognize components of MTB are given in Table 3.

Table 3. Examples of consequences by engagement of various pattern recognition receptors (PRRs)

PRRs	MTB ligand	Pathway	Cytokine spectrum/ other effects	Ref.
Scavenger receptors				
MARCO	trehalose dimycolates	with TLR2	Stimulate the formation of foamy macrophages	[4]
C-type lectins				
DC-SIGN	mannose-capped lipoarabinomannan (LAM)	RAF-1	IL-10	[97, 98]
Dectin-1	MTB glycolipids	CARD9	IL-1β, IL-6, IL-12p40, IL-17A, IL-23, TNFα	[99, 98]
Mincle	trehalose dimycolates	Syk-FcRg-CARD9	IL-1β, IL-6, IL-12p40, IL-12p70, IL-23, TNFα	[99]
Toll-like Receptors membrane-bound				
TLR2	MTB lipomannan	My88D-IRAK-NFκB activation or TRAF6-MAP	IL-12p40, IL-12p70, IL-23, downregulation of MHCII	[97, 99]
TLR4	Liopteichoic acids	with MD-2 and CD14. RAF-1		[97, 98]
TLR9			IL-12p40	[99]
intracytoplasmatic				
NOD2/CARD15	MTB mycolylarabino-galactan peptidoglycan		IL-12p40, TNFα	[97, 99]

Depending on the PRR engaged, binding induces 1) enhanced phagocytosis (mannose-binding lectins and scavenger receptors), 2) NFκB-activation and secretion of cytokines and chemokines (Toll-like receptors) and/or 3) up-regulated expression of MHC class II and co-stimulatory molecules required for antigen-presentation to lymphocytes. In the alveoli, an additional macrophage subset; residing alveolar macrophages provides a first line of defense through pathogen capture before epithelial invasion.[96]

Epithelial cells contribute by secretion of neutralizing antimicrobial peptides. In addition, they respond to direct- or indirect pathogen-induced damage by secreting inflammatory mediators capable of increasing the activation status of adjacent macrophages and DCs. Furthermore, activated by pathogenic products and/or the inflammatory state itself, the endothelial cells of pulmonary capillaries start expressing selectins (P-selectin and E-selectin) and integrins (ICAM-1, ICAM-2) required to recruit more leucocytes from the blood to the infected tissue.[95]

Neutrophils are the first innate immune cells to arrive from blood.[2, 95] Neutrophils possess anti-mycobacterial properties and can kill extracellular bacteria directly by reactive oxygen species,[95] or indirectly by empowering macrophages through degranulation of proteolytic enzymes.[100] Neutrophils are attracted to the infective site in the lung dependent on secretion of IL-8 (CXCL8) by macrophages and/or endothelial cells.[95, 101]

The next cells to be recruited from the blood are monocytes which differentiate into tissue macrophages or DCs depending on the cytokine milieu (M-CSF or GM-CSF and IL-4, respectively).[95]

Activated endothelial cells become leaky, permitting plasma proteins to enter the tissue. Plasmatic precursor enzymes of the complement system directly (C1q) or indirectly (C-reactive protein or Mannose-binding lectin) activated by pathogenic products can initiate the complement cascade further amplifying the recruitment and efficiency of immune cells.[95] Altogether, these early events of innate immunity produce a range of inflammatory mediators at the infectious site which subsequently shapes the adaptive immune responses as described in the next section. The inflammatory phenotype at the infectious site is thus critical for the efficiency and success of adaptive immune responses to prevent overt TB following MTB-infection.

1.2.2.2. Induction of adaptive immune responses

Activation of DCs residing in the lung parenchyma through PRRs, enhance their migration to the draining lymph nodes[102] where the DCs initiate adaptive immune responses by their interaction with T- and B-cells (Figure 1). Antigens presented by incoming DCs on MHC class I or II molecules are recognized by CD8+ and CD4+ T cells, respectively, specific for the antigen presented.[95] Given the intra-vesicular residence of MTB in arrested phagosomes, presentation by the conventional vesicular pathway is straight forward,[14, 103] and can also be induced by IFNγ-induced autophagy,[104] CD8+ T cells are activated by MHC class I presentation of cytoplasmatic antigens. Intra-vesicular MTB proteins reach the MHC class I loading compartment in infected or bystander APCs by cross-presentation.[105-109] CD4+ T cells differentiate into distinct functional subsets depending on the activation status of the

DC and the surrounding inflammatory phenotype (see section 1.2.3), demonstrating the importance of the innate immunity on the ensuing adaptive immune response.

B cells recognize a part of the three-dimensional structure of un-processed antigen by the B-cell receptor (BCR). Cross-linking of BCRs on the membrane or help from CD4+ T cells (T helper cells) mediated by TCR recognition of antigen presented on a B-cell MHC II molecule, is required for activation of B cells.[95]

The recognition of antigen by T and B cells triggers their differentiation and proliferation. Mature effector lymphocytes exit the lymph node through the efferent lymph and reach the circulation through the thoracic duct. Specific homing properties acquired by lymphocytes activated in the lymph node enables their migration back to the infected lung[96] (Figure 1). At the infectious site, effector T cells combat the bacteria in several different ways depending on the subset (see section 1.2.3). B cells differentiate into antibody-producing cells, designated plasma cells, that mediate humoral immunity by the secretion of antibodies. The arrival of effector lymphocytes at the infective site represents the onset of targeted and efficient adaptive immune responses.[95]

Importantly, long-lived T- and B cells are generated in parallel to effector lymphocytes during their differentiation in the lymph nodes. These cells, called memory lymphocytes, continue to circulate after the eradication/quiescence of the infectious agent providing the host with immunological memory. By re-exposure to the same antigen through re-infection or re-activation, memory lymphocytes assure a rapid generation of effector lymphocytes termed a secondary immune response that often prevents clinical disease.

1.2.3. Differentiation of T cell subsets and their mode of action

Following antigen recognition, naive CD4+ T cells differentiate into functionally distinct phenotypes (Th1, Th2, Th17 or Treg) depending on the activation state of the antigen-presenting cell (APC) and the cytokine milieu[13, 95, 99] (Figure 2).

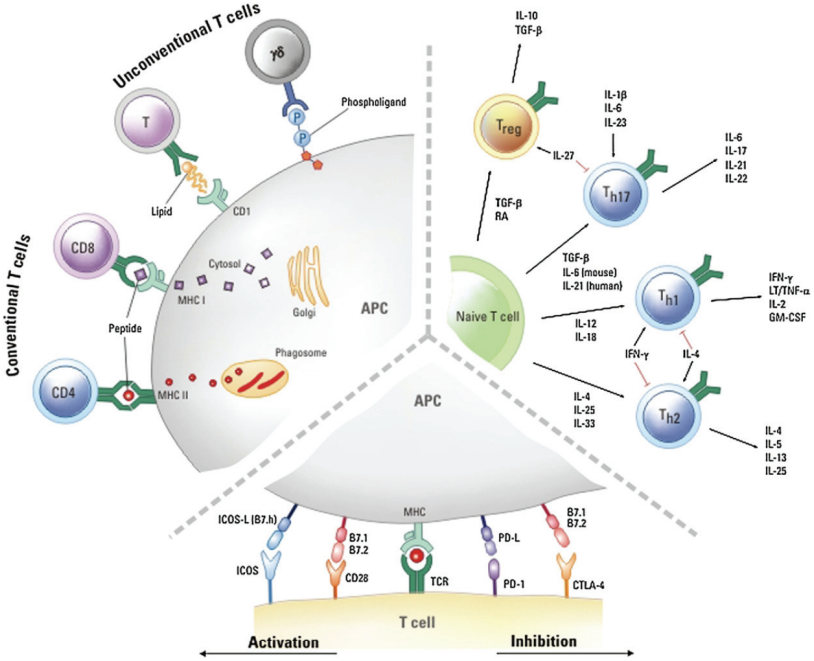

Figure 2. Presentation and recognition of MTB antigens, and factors involved in the differentiation of T cell subsets.

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In primary infected mice, it takes about 20 days before effector T cells are generated in sufficient numbers to limit bacterial growth.[110] In humans, the kinetics of effector responses in the lung after MTB exposure is not easily assessed.

1.2.3.1.CD4+ T cells

The requirement of CD4+ T cells in host protection to MTB is well established in various mouse models,[79] in cynomolgus macaques[111] and in humans in the context of HIV-infection, which is the most powerful risk factor for TB at a population level.[32, 52] Still, the mechanisms behind the protective effect of CD4+ T cells are not fully understood.[79] The discovery of CD4+ T cell subsets which differ in functional capacity necessitates a description at the subset level:

CD4+ Th1 cells

APCs with an activated phenotype secreting IL-12, IL-18 and IL-27, favor activation of the transcription factor T-bet required for differentiation of Th1 CD4+ T cells.[95, 112]

In the setting of MTB-infection, IL-12p40, the product of MTB antigen ligation with different PRRs, is required for Th1 development.[113] Th1 cells are defined by their secretion of Th1 cytokines, notably IL-2, IFNγ, TNFα and GM-CSF.[95] IFNγ and TNFα are required for macrophage activation and are both crucial in host protection against MTB[13, 14, 103] as activated macrophages restrict MTB replication by various mechanisms[103] which eventually overcome the phagosomal arrest[104, 114].

The importance of Th1 cells in human MTB infection has been demonstrated by various gene deficiencies in the cytokine-mediated macrophage activation pathway.[83, 115] Th1 cells co-producing ≥2 cytokines (polyfunctional T cells) are more potent cytokine producers,[116] and have been suggested to provide superior protection against TB progression in infected subjects.[13, 117, 118] Therefore, vaccine candidates are evaluated by their capacity to induce polyfunctional T cells.[119-121]

CD4+ Th17 cells

The differentiation and proliferation of Th17 cells is dependent on IL-6 or IL-21 in the presence of low amounts of TGF-β, which induces the transcription factor RORγt, both required for the expression of IL-23 receptor and hereby sustained Th17 responses.[99] Th17 cells secrete IL-17, IL-21 and IL-22,[122] IL-17 trigger release of IL-8 (CXCL8) and GM-CSF from endothelial and epithelial cells required for neutrophil expansion and recruitment[101, 123] probably important for the initial granuloma formation[124, 125], (see section 1.2.2.1).

Evidence suggests a protective role for Th17 cells in the early mycobaterial host defense: Vaccines inducing Th17 memory cells in the lungs induce IL-17 dependent CXCR3 ligands (CXCL9, CXCL10 and CXCL11) which accelerate the recruitment of MTB-specific Th1 cells resulting in reduced bacterial burden in WT compared to IL-17 deficient mice.[110, 126] Th17 cells/IL-17 also seem to protect against MTB infection by mechanisms independent of IFNγ.[127, 128] Once the Th1 response is adequate, the cytokine milieu will favor further Th1 delineation and suppress Th17 delineation.[99]

MTB-specific Th17 cells in TB patients predominantly express either IL-17 or IL-22 (few co-producers), unlike Th1 cells which mostly display an effector phenotype, Th17 cells display a central memory phenotype.[129] In progression to TB, Th17 cells

might no longer be protective, as IL-17 is associated with severe neutrophil inflammation in the human lung.[130] Supporting this, mice repeatedly exposed to mycobateriae exhibit necrotic lesions rich in neutrophils[131-133] Th17 cells seem less susceptible to suppression by regulatory T cells (defined and discussed in the next paragraph) than Th1 cells *in vitro*,[134] and it has been suggested that a skewing in the Th1/Th17 balance towards Th17 cells, promotes neutrophil inflammation, tissue necrosis and progression in TB pathology.[79]

CD4+ regulatory T cells

CD4+ T cells expressing the transcription factor FoxP3 are termed regulatory T cells (Tregs).[135] These cells seem crucial in limiting autoimmune diseases[136] and tissue damage following acute infections.[135] Tregs act directly (TCR-ligation) or indirectly (alterations in the cytokine milieu) on the delineation and/or activation of other T cell subsets.[137] FoxP3+ Tregs consist of 2 subsets: 1) Natural Tregs induced in the thymus by appropriate TCR-recognition and affinity of self-antigen (potential of cross-reactivity with microbial antigens), and 2) Induced Tregs which arise when FoxP3 is induced in conventional T cells in the periphery (favored by chronic antigen stimulation, limited co-stimulation and TGF-β).[138] Induction of Tregs by MTB might represent a pathogen evasion strategy for the pathogen[3, 137, 139] as Tregs reduce the induction of protective effector T cells by a contact-dependent inhibition of APCs (CTLA-4, LAG, NRP1 and surface-bound TGF-β).[140]

In early MTB infection, there is an unspecific recruitment of Tregs to the lung which result in reduced Treg numbers in peripheral blood.[135, 141] Tregs are present within granulomas.[142] There is abundant evidence from *in vitro* and *in vivo* models to support that Tregs expand during MTB infection and subsequently reduce and delay protective effector T cell responses.[3] The effect seems to be mediated by natural Tregs specific for MTB antigens by cross-reactivity.[135] Pre-existing specific Tregs generated by oral exposure to NTMs could have an impact on vaccine take and the delayed induction of adaptive immunity. This delay likely has an impact on bacterial load and subsequently the resistance to TB progression.[79]

In TB patients, there is a positive correlation between bacterial load/immunopathology and Treg numbers, but whether Treg expansion in this setting is a cause or a consequence of TB disease is still unknown.[135]

FoxP3 negative CD4+ T cells with regulatory properties through their cytokine secretion, can also have an impact on the host immune response to TB,[143-147] but are not further discussed here.

1.2.3.2.CD8+ T cells

Following TCR-ligation, MTB-specific CD8+ T cells restrict bacterial growth by granulysin- and perforin mediated apoptosis of MTB infected macrophages or by macrophage empowerment through IFNγ and TNFα secretion.[14, 103] No data support a non-redundant role of CD8+ T cells in the control of early or latent MTB infection [1], but their importance might increase in later phases of infection[148] when IFNγ-secretion dominates compared to the cytotoxic activity.[149] Some results indicate that CD4+ T cells might be required for the expansion of MTB-specific CD8+ T cells.[111]

1.2.3.3.Prolonged T cell responses

Rapid expansion and maintenance of short-lived effector T cell pools (CD4+ T including Tregs, and CD8+) depend on IL-2.[150] In a state with continued exposure to antigen, as in MTB infection, IL-2 seems to be produced mainly by effector memory T cells (T_{EM}), but IL-2 production and the ability to replicate is lost when the cell further differentiate towards terminal effectors (T_{TE}). Therefore, without the generation of long-lived central memory T cells (T_{CM}) which exhibit high proliferative capacity by repeated antigen exposure, the pool of effector T cells cannot be sustained.[13, 117] Effector T cell responses are also self-limiting through IFNγ- and TCR dependent up-regulation of inhibitory molecules (CTLA-4, PD-1) and down-regulation of co-stimulatory molecules, which result in T cell inhibition and apoptosis.[5, 151] MTB-specific T cells in TB patients are predominantly T_{EM} cells,[152] and some suggest that progression to TB is proceeded by exhaustion of the memory T cell pool by sustained exposure to MTB antigens.[13, 117, 153] Progression to TB might thus be a consequence of waning immunity.

The generation of T_{CM} is the goal of vaccination, as these subsets ensure rapid deployment of high-affinity adaptive immunity at primary or secondary exposure to pathogen (due to re-exposure or re-activation) [13]. IL-7 and IL-15 are crucial in the generation of memory T cells.[150, 154] T_{CM}, T_{EM} and T_{TE} have different homing capacities and can therefore be distinguished by phenotypic markers (Table 3). [155, 156]

Table 3 **Markers of T cell differentiation**

	marker	naïve	central memory (CM)	effector memory (EM)	terminal effector (TE)
T cell differentiation	CD45RA	+	-	-	+/-
	CD45RO	-	+	+	-
homing to lymph node	CCR7	+	+	-	-
	CD62L (L-selectin)	+	+	-	-
activation marker	CD27 (TNF-receptor family)	-	+	+	-
?	KLRG1	?	-	-	+

1.2.4. How MTB interferes with host immune responses

The downstream events induced by antigen recognition depend on the affinity of the PRR:MTB ligand-binding which is subject to genetic variation in both host and pathogen. This could be factors which contribute to the observed differences in host susceptibility and MTB virulence.

Being incorporated in early endosomes, MTB are targeted for intravesicular destruction within the engulfing macrophage, but MTB has evolved to prevent phagosome maturation and endolysosomal fusion arresting phagosomal degradation. Within this arrested phagosome MTB ensures the acquisition of nutrients (iron and lipids) within the arrested phagosome, by interfering with intracellular trafficking pathways.[14, 157, 158] By now, MTB have exploited the phagocytic properties of an alveolar macrophage to establish a protected niche for replication until adaptive immune responses are put into play.[79] Evidence suggests that MTB interferes with additional properties of the host macrophage:

- inhibits IFNγ-activated genes and antigen-presentation.[159-161]
- inhibits autophagy which can kill MTB regardless of the phagosomal arrest (induced by IFNγ, inhibited by IL-4 and IL-13).[104, 162]
- increase the production of anti-inflammatory cytokines.[163]

- favors necrosis to apoptosis of infected macrophages either through inhibition of TNFα-dependent apoptosis,[164, 165] membrane repair[166] or interference with the eicosanoid metabolism.[167] This delays the DCs uptake of MTB and their subsequent activation and migration.[168]
- exaggerated production of IFNγ and nitric oxide (NO) which subsequently lead to suppression of cell-mediated immune responses.[169]

These examples illustrate how MTB subverts host immune responses by promoting the production of anti-inflammatory cytokines and delaying activation and antigen-presentation by DCs, permitting bacterial replication within the macrophage. *In vitro* studies suggest that MTB is unable to prevent endolysosomal fusion in pre-activated macrophages,[158] but the anti-inflammatory alveolar macrophages are probably easily subverted. Furthermore, in a setting with both resting and activated macrophages, MTB seem to inhibit uptake by activated macrophages by mannose derivatives on the surface.[170]

Macrophages seem to undergo necrosis at a certain threshold of bacterial load resulting in the release of viable bacilli which can infect neighboring mononuclear cells[171] and neutrophils.[172] Studies in mice suggest that a mechanism for genetic susceptibility to MTB is an increased tendency of infected macrophages to undergo necrosis instead of apoptosis.[173] Importantly, IFNγ can reverse some of these effects.[104, 114] MTB also seem to impose changes in uninfected bystander DCs through shedding of immune-modulating antigens in apoptotic blebs.[107, 108]

MTBs immune modulating properties together with the slow replication rate keeps the inflammation down, but either a high inoculation dose or release of viable bacilli following macrophage necrosis, will sooner or later activate other players of innate immunity and induce an inflammatory reaction.

The onset of adaptive immune responses is delayed in MTB infection compared to other infections[3, 174] (Figure 3).

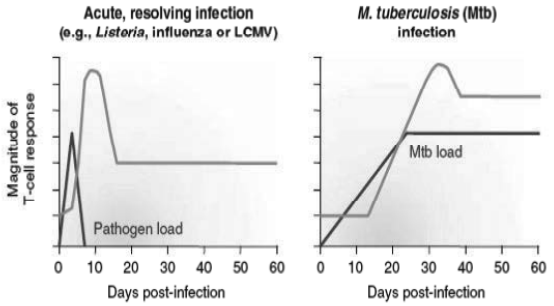

Figure 3. The adaptive immune responses are delayed in MTB infection compared to other infections.

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This is likely due to insufficient activation and migration of DCs due to multiple factors: 1) the regulatory environment in the alveoli (alveolar macrophages and tolerogenic DCs),[3, 96] 2) the slow MTB replication rate,[6] 3) the capacity of MTB to suppress inflammation by a) inhibition of co-stimulatory molecules and induction of anti-inflammatory cytokines[79, 163] or b) Treg activity[135] (Regulatory T cells in MTB infection; section 1.2.3.1). How the DCs capture antigen also seems important: by phagocytosis of free MTB bacilli (after necrosis of infected macrophages)[109] the DC itself will become infected and exhibit reduced antigen-presenting capacity,[102] whereas engulfment of MTB containing vesicles from live or apoptotic macrophages will leave the DC uninfected and retain their antigen presenting capacity.[109] In the setting of MTB infection, the importance of migrating DCs is unclear as dissemination of viable bacilli to the draining lymph nodes seems required for induction of adaptive immunity in mice. This dissemination occurs 8-9 days after challenge and corresponds to necrosis of infected macrophages.[174-176] In mice with primary infection, it takes 15-18 days post infection before MTB-specific T cells are present at the primary site.[175] Thus MTB delays the kinetics of effector T cell proliferation and migration by altering the DCs phenotype as described above, and a direct effect of induced MTB-specific regulatory T cells[135, 177] (Regulatory T cells in MTB infection; section 1.2.3.1.).

1.2.5. Granuloma formation – a result of the host-MTB interplay

1.2.5.1.Early granuloma formation

Sooner or later infected alveolar macrophages start secreting TNFα and other inflammatory chemokines (CCL2, CXCL10, IL1-α, IL1-β, IL-18).[2] As described, neutrophils and monocytes are recruited first, and become important sources of TNFα and CXCR3 ligands required for T cell recruitment and granuloma formation.[125, 178]

The arrival of MTB-specific lymphocytes contributes to the broad production of cytokines and chemokines (TNFα, CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL4, CCL5). Adaptive immunity is increasingly put into play, a mounting IFNγ-production provides a negative feed-back which will gradually down-regulate the pro-inflammatory innate responses (Figure 4). The net result is rearrangement of the micro-anatomy at the infectious site to a granuloma[2] in which MTB is confined within a hostile environment exposed to NO, CO and low pH. In response to this, MTB up-regulates 1) the DosR-regulon, which constitutes 48 genes which induces a dormant or slow-replicating state[179, 180] and other transcriptional regulators which result in 2) nutrient-switch from glucose to fatty acids/cholesterol and 3) increased fortification[4] to ensure its survival. Notably, these dynamics in the MTB gene expression profile is likely to affect the MTB antigens available for T cell recognition.[5]

With lack of CD4+ T cells in the context of HIV/AIDS or in mouse models, the immunopathology of MTB infection is shifted from granulomatous lesions to neutrophil inflammation and necrosis.[132, 181] Even though neutrophils might provide protection in early MTB infection, their inflammatory properties might enhance MTB-related pathology at later stages.[99] Recently, Berry et al, have provided evidence which indicate that neutrophils are more important in TB pathogenesis than earlier anticipated. The transcriptional profile of peripheral blood of TB patients was dominated by up-regulation of both IFNγ and type I IFN-inducible transcripts, to which neutrophils contributed the most.[182]

Findings of MTB-containing APCs surrounded by proliferating B cells and T cells in the outer mantel of lung granulomas, suggest the formation of secondary lymphoid structures.[183, 184] B-cell deficient mice are more susceptible to TB disease[185] and have more severe immunopathology with increased neutrophil accumulation,[186] effects which might be mediated by Fcγ-receptors.[187] Based on this, B-cells are suggested to play a role in the formation and maintenance of granulomas.[79] In humans, a recent study reported a reduction in B cell transcripts in peripheral blood of TB patients.[182]

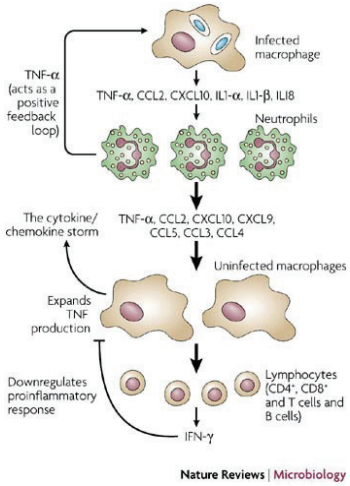

Figure 4. The successive recruitment of immune cells is driven by cytokines and chemokines secreted by the infected macrophage.

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1.2.5.2.Evolution of the granuloma and emergence of overt TB

The structure of the initial human granuloma is highly vascularized but if it develops further, the vascularization decreases and the core of MTB infected macrophages becomes increasingly surrounded by different morphotypes of differentiated macrophages (giant cells, epithelioid cells and foamy macrophages) and a fibrous layer which physically seem to isolate MTB from the surrounding mantle of MTB specific CD4+ and CD8+ T cells. Subsequently, excessive granuloma formation ensures caseation, necrosis and MTB transmission[4] (Figure 5).

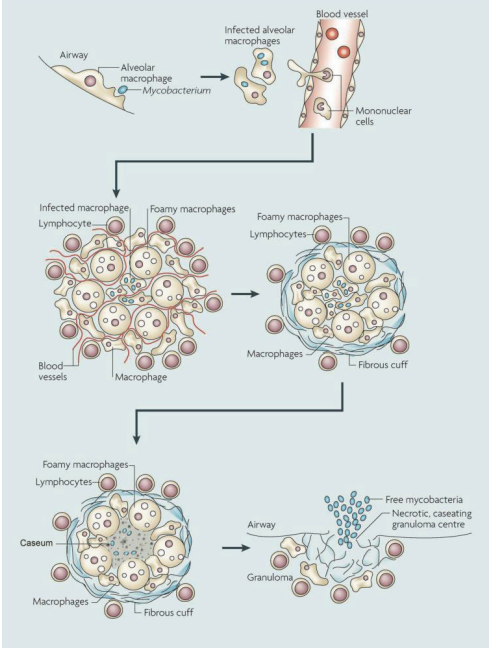

Figure 5. The formation and destabilization of the human tuberculosis granuloma.

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In humans and non-human primates (NHP), tubercular granulomas exhibit a broad spectrum of histological and functional features within the same individual.[2, 188] Furthermore, repeated CT/PET-scans in NHPs and human TB patients provide evidence that granulomas are highly dynamic and act independently from each other[1, 189] (personal communication, Clifton E Barry at the Keystone Symposia "Host Response in Tuberculosis", March 13-18, 2013, Whistler, BC, Canada). Whether the integrity of the granuloma persists or destabilizes, resulting in caseation, seem to be determined locally by a complex interplay of host chemokines, cytokines, immune cells as well as MTB characteristics.[1, 4, 178, 190] The mechanisms involved are not yet fully understood, but the bacterial load within the granuloma seems to be an important factor in granuloma destabilization.[79, 189] Furthermore, MTB-bacilli are localized in different parts of the granuloma; from the necrotic center to outside the fibrotic capsule. Depending on the localization, the balance between the pathogen and the host immune response is likely to represent a broad spectrum: while bacilli might be kept dormant or slow-replication outside the fibrotic capsule of the granuloma, other bacteria in the granulomatous center could be replicating.[1, 2] The determinant factors/mechanisms in granuloma destabilization and progression to overt TB are not fully understood but might include:

- *Oxygen restriction* facilitates breakdown of the granulomatous structure to caseation and necrosis. This is consistent with reduced vascularization and levels of the macrophage-derived vascular epithelial growth factor (*VEGF*) in caseous granulomas compared to non-progressive granulomas[2].
- Recent evidence suggest *that MTB and/or hypoxia induces matrix metalloproteinases (MMP)* which induces collagen destruction and drives tissue pathology in TB.[191, 192]
- Some argue for a role of MTB induced *pro-inflammatory foamy macrophages,*[4] which so far have been identified in necrotic lesions only.[4, 193]
- A shift from *apoptosis to necrosis of infected macrophages at a certain bacterial load* could be an important mechanism[171] and provides an argument that MTB are slowly replicating rather than fully dormant inside the granuloma-residing macrophage. The threshold is likely to be dependent of MTB strain and a number of host factors.[171, 173]

- *A shift in the balance of CD4+ T cell subpopulations (Th1/Th17/Treg)* promoted by factors in the microenvironment might proceed increased inflammation and progression to granulomatous caseation and necrosis. A simplified hypothesis build on the documented plasticity of T cell subsets: Tregs can be converted into Th17 cells by pathogen components and IL-6[194] and steadily increasing bacterial load could be permitted by the presence of Tregs impairing Th1 function. Alternatively, increased bacterial load could have a broader impact on the cytokine milieu through DC or MMP-mediated tissue-destruction and hereby the delineation of CD4+ T cells. Once Th17 cells have reached a certain level (permitted by local IL-23[99]), they become resistant to regulation by Th1 cell[133] and the vicious cycle of neutrophil inflammation and tissue destruction accelerates.

To summarize, the granuloma is the hallmark of established MTB-infection, and actually represents a favorable outcome for both host and pathogen.[4] For the host, the granuloma confine MTB in a hostile environment required for initial bacterial control whereas lack of granuloma formation results in bacterial dissemination and rapid disease progression. For MTB, persistence within the granuloma exhibits a potential for caseation and necrosis - a niche for later replication, bacillary spread and transmission.[2, 79] The emerging consensus is that MTB infection result in a broad spectrum of pathological manifestations closely related to the balance between MTB and the host immune response. TB occurs when the pathology result in symptoms (Figure 6).

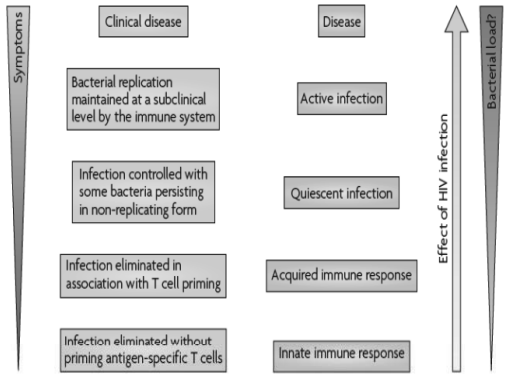

Figure 6. The inter-relationship between bacterial load, host immune responses and clinical manifestations.

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The rapidity in the onset of efficient adaptive immune responses following infection is crucial in controlling bacterial replication. The bacterial load at the time of equilibrium between host immune responses and MTB (containment inside a granuloma) seems to be a crucial determinant for the fate of the individual granuloma and the subsequent risk of TB progression in the host.[79, 176]

1.3. Readouts of the host immune response

As indicated above, the host immune response towards MTB is tightly linked to the immune-pathology. Therefore, following a global initiative for biomarker discovery[49], different components of the host immune response to MTB have been extensively studied for their potential as diagnostic and prognostic biomarkers.[195] A biomarker marker is *"a characteristic feature that is objectively measured and evaluated as an indicator of a normal or pathological process or of the response to intervention.* [196] (MTB derived biomarkers have been studied in parallel, but are not a subject for this thesis.) In MTB infection, single biomarkers seem to be of little value, whereas a profile of combined biomarkers, termed a biosignature, is probably more appropriate due to the complexity in host immune response.[195]

The selection of specimens for biomarker studies depending on disease localization, availability and risk related to sampling. Peripheral blood has obvious advantages when it comes to availability and risk compared to other specimen (sputum, broncho-alveolar lavage (BAL), gastric aspirate, tissue biopsy). Invasive procedures in the diagnosis of MTB infection in absence of active TB are hardly ethical. Evidence suggests that biomarkers in peripheral blood reflects environmental, physiological and pathological events occurring in different tissues of the body,[197] and this is supported by correlative data on transcriptional biomarkers and the radiological extent of TB disease.[182] Peripheral blood has also been regarded appropriate when it comes to T cells; the hallmark mediators of protective immunity towards MTB, as they circulate in peripheral blood prior to recruitment to the site of infection.[53]

Biomarker discovery can be assessed by un-biased approaches of gene signatures (micro-array/transcriptomics), protein signatures (proteomics) or metabolic signatures (metabolomics) or by analysis of specific transcriptomes, proteins or metabolic

metabolites, or cell populations pre-selected because of their documented role and relevance in the host immune response to MTB.[5, 198] Analyses are performed both direct *ex vivo* (unstimulated) or after *in vitro* stimulation depending on the approach.

1.3.1. The need for TB biomarkers and improved diagnostics

Biomarkers in TB are crucial in the development of new therapeutics and vaccines and current research focuses on four areas:[5, 31, 195, 199]

1. Improve the diagnosis of active TB.
2. Prediction of treatment efficacy and cure.
3. Prediction of reactivation/progression to TB in MTB infected healthy subjects.
4. Prediction of protective immunity by vaccine candidates/surrogate end-points in vaccine trials.

In children <3 years the need for better tools in the diagnosis of MTB infection and active disease is urgent. With regard to MTB infection, children this young have increased risk of rapid and more severe TB manifestations[12] which have led to recommendations of preventive treatment.[58] With regard to active disease, adequate management is challenged by a high proportion of unconfirmed cases (70-80%),[75] as sputum negative TB cases are not prioritized for treatment by many national TB control programmes. TST and QFT are used both to diagnose MTB infection and as supplements in the diagnosis of pediatric TB[12, 71, 200] despite proper validation in young children.[63, 64, 201] Data on TST and QFT performance in different cohorts of young children are needed in order to ameliorate the interpretation of test results. In addition, new biomarkers have the potential to increase the diagnostic accuracy and thereby contribute to a better management of TB in this vulnerable age group.[31, 71, 200] Notably, a rapid biomarker-based, instrument-free test for non-sputum samples that also detects childhood and extrapulmonary TB, ranked second in recent ranking of diagnostic needs.[202]

For biomarkers to become true tools in the global management of TB, they need to be confirmed in populations heterogeneous with regard to genetics, endemics of HIV, mycobacteria and helminthes as well as socioeconomic determinants.[195]

2. AIMS OF THE STUDY

The general aim of this thesis was to contribute to a better understanding and interpretation of immunological tests developed to diagnose *Mycobacterium tuberculosis* infection, and to explore the diagnostic potential of new immune biomarkers in *Mycobacterium tuberculosis* infection and disease in children.

2.1. Specific aims

Paper I: To evaluate the performance of TST and QFT, and their association with clinical, demographic and nutritional (intra-uterine and post-natal) characteristics in a cohort of BCG-vaccinated young children in Southern India.

Paper II: To explore the diagnostic potential of new immune biomarkers in *Mycobacterium tuberculosis* infection and disease in BCG-vaccinated young children in Southern India.

Paper III: To characterize immune biomarker profiles associated with concordant or discordant TST and QFT results in BCG-vaccinated young children in Southern India.

Paper IV: To explore the quality and/or magnitude of mycobacterial-specific T-cell responses associated with QFT reversion and persistent QFT-positivity in adolescents in Southern India.

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3. MATERIALS AND METHODS

3.1. Source population

The source population of this study was ~ 400 000 inhabitants in Palamaner Taluk (3.200^0 N, 72.7500^0E, altitude 683 m) in Chitoor district, Andhra Pradesh, Southern India (Figure 7) from April 2007 to September 2010. In India today, the estimated annual incidence of TB disease is 176/100 000, and the HIV prevalence is 0-4%.[51] The incidence of TB in the state Andhra Pradesh was 136/100 000 in 2010.[203]

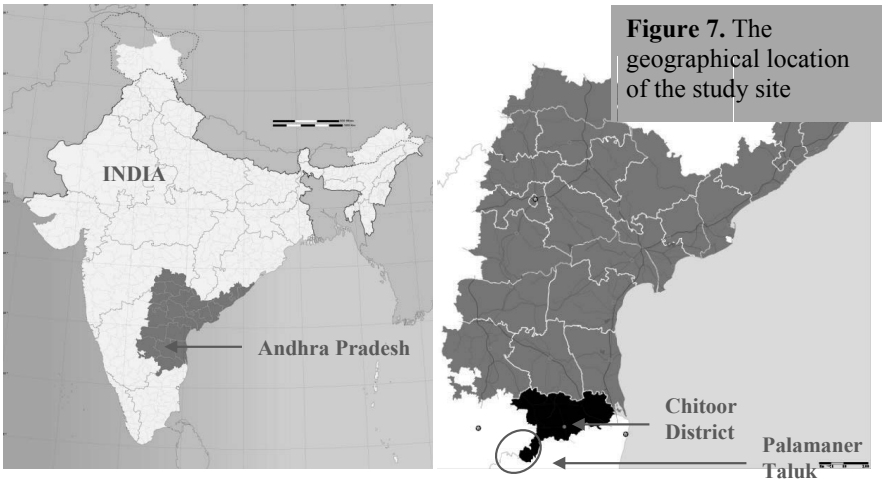


Figure 7. The geographical location of the study site

Palamaner Taluk consists of 7 community units ("mandals") comprising 594 population units of discrete villages and town with a minimum population of ~200 subjects. The district is largely rural and semi-urban and the majority of the population is involved in agriculture or related activities.

In India, BCG vaccination is advocated at birth, and the vaccine coverage during the study period has been estimated to be 87%, but was probably 70-74% in the period when the adolescents eligible for our study were born.[204] Prior to the study set-up, the BCG coverage in the study area was determined to be about 85% before the age of 9 months (Mario Vaz, Indian Principal Investigator for the TB Trials, St. John's Research Institute,[205] Bangalore, personal communication).

3.2. Study populations, enrollment, randomization and data collection

The study participants included in this thesis are drawn from the study population of the Neonatal Cohort Study (NCS) and the Adolescent Cohort Study (ACS). These prospective population-based observational cohort studies were set up in the source population described in section 3.1, as part of the process for preparing Palamaner as a field site for future TB vaccine trials. Conducted by the TB Trials Study group (contributors listed in the papers), NCS and ACS were designed to establish the incidence of TB disease during a 2-year follow-up period of

- children in one annual birth cohort (NCS) and
- randomly selected school clusters of adolescents aged 12-18 years (ACS).

A field study site was established at Emmaus Swiss Hospital, coordinated from the St. John's Research Institute,[205] funded by AERAS[206] and the Research Council of Norway (TB Trials 179342 and 192534). All clinical TB cases identified during the studies, were referred to the TB public programme[203] for initiation of anti-TB treatment.

Families who planned to move from Palamaner during the follow up period were not eligible. Written informed consent was obtained from participants aged >18 years or from each participant's legally authorized representative. In addition, a written assent to participation was obtained for adolescents aged <18 years. The consent process in the NCS study is described and evaluated by Rajaraman et al.[207]

Data were captured in clinical research forms and entered into a database following Good Clinical Practice (GCP) guidelines.[208] Further inclusion criteria, data collection and sampling of specimens are described below, separately for the NCS and the ACS. Individual HIV status was not assessed as part of the NCS or ACS, but data from a house-hold contact study conducted in the same study area in the period 2010 to 2012, found a HIV prevalence <1% (TB Trials Study Group, unpublished data).

3.2.1. Neonatal Cohort Study (NCS) (paper I-III)

3.2.1.1. Enrolment

Neonates were eligible if BCG vaccine was administered by study staff within 72 hours of birth and written informed consent was obtained within 15 days of birth. The BCG strain used by public health facilities in the study area is a strain originating from Danish 1331, manufactured by either the BCG Laboratory in Guindy South India or the Serum Institute, Pune. Of 7 424 women identified antenatally in the study area, 4 872 fulfilled the inclusion criteria (eligible population). The majority of neonates that did not meet the inclusion criteria were not available during the 15 days post-delivery since it is common practice for women to travel to their mothers home to deliver. Of the eligible population, 4 382 (89.8%) were enrolled in the NCS. Parents were provided a Study Identification Card with the study physician's contact details, to keep track of important health information, and were encouraged to contact the study office if their child was ill, exposed to a known TB case, diagnosed with TB, admitted to hospital or died. In addition, all primary health centres and referral hospitals within and around the study area, were visited weekly by study staff to determine if any study participants were admitted or treated with a presumptive diagnosis of TB or related diagnoses like HIV/AIDS.

3.2.1.2. Randomization and follow-up

The 550 population units were first stratified according to the type (semi-urban; rural, high development, rural, low development) and the size of the population (<500, 500-1000, >1000). This stratification was done because the TB incidence is likely to be different between these communities. Population units within each stratum were subsequently randomly assigned into active or passive surveillance. Thus, all neonates within a population unit were assigned to the same surveillance group. This was to avoid cross-over between the surveillance groups in the follow-up process.

- Infants with *active surveillance (2 215)* had bimonthly home visits by a study team member.
- Parents of infants with *passive surveillance (2 167)* received health education about TB symptoms at enrolment, and were encouraged to bring their children to the case verification ward (CVW) if they developed such symptoms. The only home visit was at study closure.

3.2.1.3.Data obtained by surveillance per-protocol

- At baseline, time of birth, clinical data of pregnancy length and delivery were registered by medical records and/or interview. Weight were measured by spring balances and height measured recumbent (lying down) on a length board and read according to standard guidelines (Figure 8). Age of the mother and socio-economic variables (parental education (6 levels), caste (3 levels), religion (7 levels), type of housing (7 levels) and cooking fuel (7 levels)) were obtained in interview by study staff.

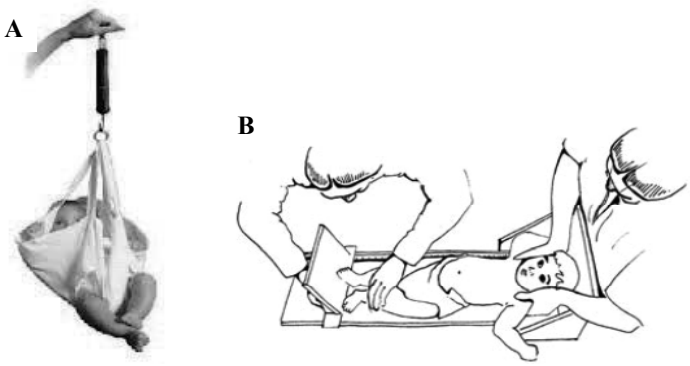


Figure 8. A) Spring balances were used for measuring child weight. The balances were calibrated with standard weights once a month. B) Correct recumbent height measurement on a length board (fixed headboard, movable foot board) requires 2 trained staff.

- Data obtained by interview at the regular per-protocol visits (see section 3.2.1.2) included known contact (>8 hours) with a TB case without or on treatment, symptoms (longstanding cough, fever and/or weight loss) and weight measurement as described above.
- At study closure, TST was performed on all study subjects.

Detailed description of data acquisition is given in section 3.4-3.8.

3.2.2. Adolescent Cohort Study (ACS) (paper IV)

3.2.2.1.Enrolment

Adolescents aged 12-18 years attending high schools or junior colleges in Palamaner Taluk, were eligible (estimated to be 17 818 students in 2006) for the enrolment study-target set to 8 000 subjects. Enrolment required written informed consent from each subject's legally authorized representative or from participants aged ≥18 years. In addition, written assent from participants aged <18 years was required.

3.2.2.2.Randomization and follow-up

The schools were stratified into public- or private schools and further sub-stratified according to size (public high schools) or type (private high school, private junior college, public junior college). This stratification was done because the TB incidence is likely to be different between the different strata. Subsequently, the schools within each stratum were randomly assigned for either

- *active surveillance (3 102)* with clinical evaluation every 3rd month and sampling of PBMCs every 6th month. QFT was included from study day 360 and repeated at day 720.
- *passive surveillance (3 542)* with clinical evaluation and sampling of PBMCs at baseline and study closure.

Adolescents with active surveillance only, were eligible for paper III.

3.2.2.3.Data obtained by surveillance per-protocol

- At baseline, gender, age, socioeconomic determinants, known TB exposure, presence of a BCG scar, previous TB disease and on-going symptoms were obtained in interview by study staff. Anthropometric measures (weight, height), the hemoglobin level and TST (repeated within 1-4 weeks if <5mm; using the larger of the two measurements) were registered. Subjects with respiratory symptoms were assessed for TB through clinical investigations (section 3.4), a TST (section 3.5), chest X-ray(s) (section 3.6) and microbiological analyses (section 3.7 and 3.8.1) A diagnosis of TB required: 1) one sputum culture positive for MTB, or 2) one AFB-positive sputum smear and one positive PCR for MTB.
- Every 3rd month (active surveillance only), data from the clinical evaluation including recent exposure to a TB patient within the house-hold or class (without or on treatment), symptoms (unexplained cough, weight loss, fever and/or night-sweats for >2 weeks, and/or haemoptysis) and anthropometrics were captured. This assessment was done on all study subjects at study closure.
- Every 6th month (active surveillance only), blood for banking of PBMCs and other exploratory immunological purposes.

- Data on TST repeated at study day 360 and 720 (study closure), and QFT (added to the study protocol during the study) at day 360/540 and 720 were registered.

Detailed description of data acquisition is given in section 3.4-3.8.

3.3. Selection of study participants and study outcomes

3.3.1. Neonatal Cohort Study (paper I-III)

During the follow-up, study staff evaluated the children within the active surveillance group with regard to defined criteria which led to referral to the Case Verification Ward (CVW) at the Emmaus Swiss Hospital:

1) more than 8 hours of exposure to a known TB case (without or on treatment) within the last year,

2) symptoms suggestive of TB (longstanding cough and/or fever and/or loss of weight)

3) failure to thrive (FTT) defined as any of the following;

a) loss of weight or no weight gain for two consecutive visits,

b) down-ward crossing of two percentile lines on the weight for age growth chart, or

c) weight that tracked consistently below the 3rd percentile in the WHO Child Growth Standards[209] or

4) a TST ≥10 mm at study closure.

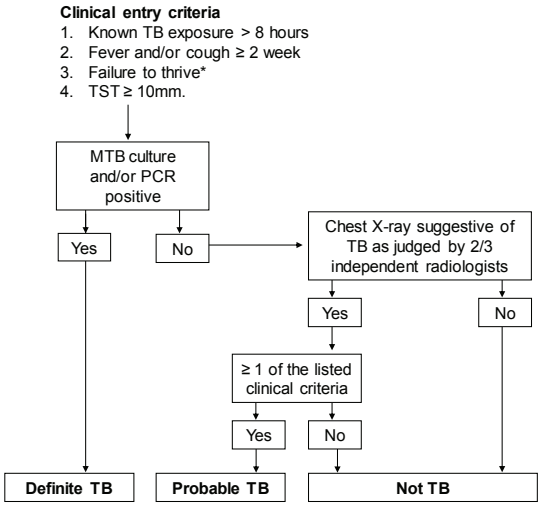
Children in the passive surveillance group were referred to the CVW by general health services informed of the NCS study, directly and/or indirectly (through the caretakers) if TB was confirmed or suspected.

All children referred to the CVW during the 2-year follow-up were eligible for inclusion in the studies described in papers I-III. At the CVW, a clinical assessment of each child was performed, with an aim of classifying the children according to pre-defined TB outcomes (Table 4).

Table 4. Outcomes/endpoints	Definition	Paper I	Paper II	Paper III
Clinical outcomes (diagnostic algorithm, Figure 9)				
Clinical TB	Definite or Probable TB according to diagnostic algorithm, Figure 9.	X	X	
Definite TB	MTB confirmed in culture or by PCR	X	(X)*	
Probable TB	symptoms and radiological changes (chest AP view) suggestive of TB	X	(X)*	
Outcomes based on diagnostic tests				
TST result	cut-off ≥10mm (pos/neg)	X		X
QFT-GIT result	cut-off ≥0.35 IU/mL (pos/neg/indeterminate[a])	X		X
MTB infection	TST+ and/or QFT-GIT+		X	

*In paper II, children with definite and probable TB was designated as clinical TB, not assessed and discussed as distinct groups. [a]Children with indeterminate QFT were not included in paper III.

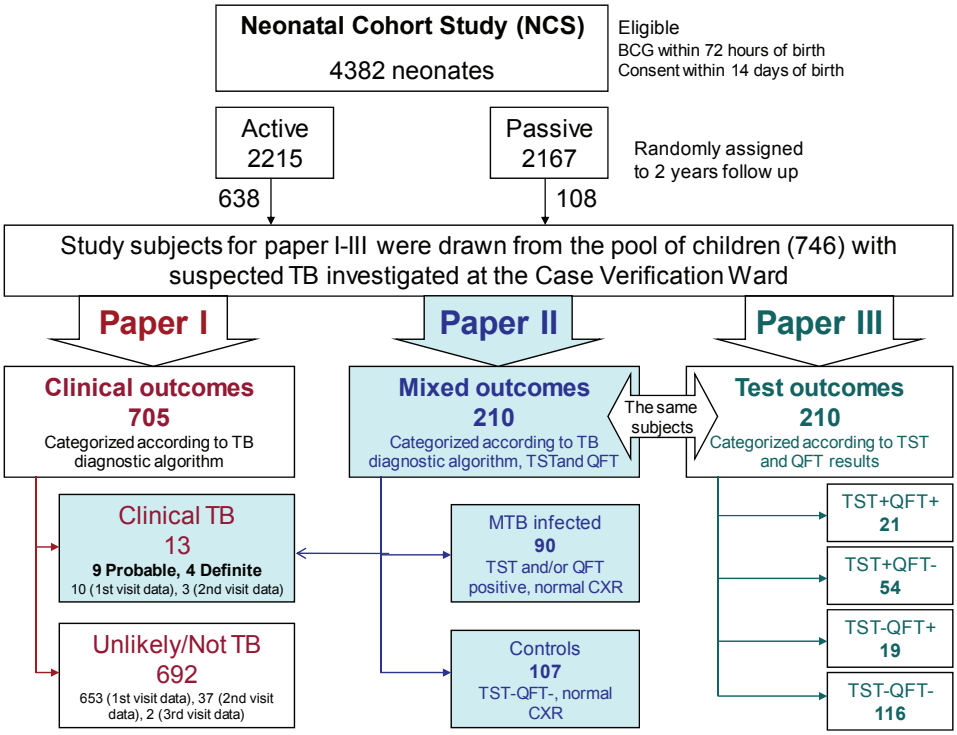
Figure 9. Diagnostic algorithm for pediatric TB. The categorization was done by the treating clinician who was blinded to the QFT result.



*Failure to thrive, defined in section 3.3.1, point 3).

TB was suspected in 746 children who accounted for 803 referrals during the study period. 38 children were referred twice, 2 were referred 3 times, and one child 4 times. According to the outcome definitions, the flowchart of study participants in paper I-III is given in Figure 10. Supplementary details for each paper are given in the text below.

Figure 10. Flowchart for study participants in paper I-III. [1]TST≥10mm, [2]QFT≥0.35IU/mL



Paper I

Of the 746 children, 705 were classified by the diagnostic algorithm for pediatric TB. Forty-one of the 746 children were not classified according to the diagnostic algorithm and subsequently excluded from the data analyses; 40 children because of lack of data from sputum/gastric aspirate, CXR and/or TST, and 1 child because he was lost to follow-up. Among the 40 children, only 1 child had known TB exposure, but the CXR in this child was normal. For children with multiple visits, the latest visit was included in the dataset as follow-up observations are highly relevant in the diagnosis of clinical TB. If QFT and/or TST were lacking at the latest time-point, data from the previous time-point was included if the clinical TB diagnosis remained unchanged (Figure 9). Visits lacking data on clinical TB category were also excluded.

Paper II

Of the 746 children, the clinical TB cases (13) as well as 90 children assumed to be MTB infected (positive TST and/or QFT and normal CXR), had adequate specimen available for biomarker analysis. In addition, 107 uninfected children were randomly selected amongst TST/QFT negative children with normal CXR and adequate specimen for biomarker analysis. The random selection of un-infected controls aimed at getting similar distribution of sex and age within the categories by age and gender matching.

Paper III

The 210 subjects selected for paper II were reclassified exclusively dependent on their TST and QFT results.

3.3.2. Adolescent Cohort Study (paper IV)

Adolescents randomized to active surveillance who did not develop TB disease during the 2-year follow-up, were eligible if they had valid QFT test results and PBMCs in storage at 2 time-points: study day 360; hereafter referred to as the QFT baseline, and 1 year later (study day 720). The adolescents were identified based on their QFT result at the 2 time-points and classified as: consistent positives, reverters or consistent negatives (controls). Then computerized random selection within the groups was undertaken (Table 5).

Table 5. Study participants in paper IV	**QFT-GIT result** cut-off ≥ 0.35 IU/mL		
	Baseline	1 year later	n
Consistent positive	+	+	21
Reverter	+	-	21
Consistent negative	-	-	10
Total			**52**

3.4. Clinical assessments (paper I-IV)

Table 6. Clinical assessments relevant to the study		NCS		ACS
		Paper I- III		Paper IV
		Surveillance		
	Description	Active	Passive	Active
When assessed				
Baseline and study-closure	per protocol	X	X	X
Every 2 (NCS) or 3 (ACS) months	per protocol in active surveillance	X		X
At the CVW*	after self-referral or referral by study staff	X	X	
Medical history				
History of TB in study subject (ACS subjects only)	TB previously suspected in study subject?	X		
Symptoms	NCS: Fever, cough or FTT**/weight loss. ACS: Fever, cough, weight loss, night sweats, hemoptysis. Duration of these symtoms	X		X
TB exposure	Known exposure to a TB patient NCS: with/without active treatment >8h. ACS: within the household or classroom	X		X
Examination				
Antropometric data	Height, weight	X		X
Examinations (only at the CVW*)				
Antropometric data	Height, weight	X		
Palpation	lympnodular enlargement, abdomen	X		
Ausculation	lung and heart	X		

*Case Verification Ward

**Failure to thrive. Defined in section 3.3.1, point 3.

3.5. Tuberculin skin test (TST) (paper I-IV)

Tuberculin Skin Test (TST) was performed by a trained nurse/doctor; 2 TU/0.1 mL tuberculin; Span Diagnostics Ltd, India were injected intra-dermally on the volar part of the lower arm and read after 48 hours. A cut-off of ≥10mm was chosen according to the Indian recommendations for childhood TB.[210]

3.6. Chest X-ray (paper I-IV)

Interpretations were done by 3 independent expert radiologists blinded to clinical symptoms and classified as 1) *normal*, 2) *abnormal not TB* and 3) *abnormal consistent with TB*. Consensus by 2/3 was required to classify the radiographic findings as *abnormal TB*.

Paper I-III (NCS)

A chest radiograph (CXR), anterioposterior (AP) view, was performed on all referred children.

Paper IV (ACS)

Adolescents with symptoms or known TB exposure at baseline had a CXR AP view performed to exclude active TB.

3.7. Sampling and handling of specimen

3.7.1. Acquisition of microbiological specimen (paper I-IV)

Paper I-III (NCS)

Induced sputum (collected in a 20 ml tube using an infant mucous extractor after nebulisation with hypertonic saline (3 %) and gastric aspirate (50ml tube containing 10% sodium carbonate) were collected in the mentioned order on two consecutive mornings for *M. tuberculosis* smear and culture (see 3.8.1.). If no sample was obtained through the mucous extractor, the child was given chest physiotherapy and the nebulisation was repeated once before proceeding to gastric aspiration. The rationale for doing induced sputum first, was that if sputum was swallowed instead of expectorated it would be recovered in the gastric aspirate. A minimum of 4 hours fasting was required before the procedures.

Paper IV (ACS)

Two sputum specimens were collected at baseline from adolescents with symptoms or known TB exposure. Induced sputum as described above was performed if naturally produced sputum could not be obtained.

3.7.2. Peripheral blood (paper I-IV)

Peripheral blood for:

- Hemoglobin (Hgb) measurements (capillary blood from heel-prick) (paper I-III)
- QFT (~3 ml, see 3.8.2) (paper I-IV)

- PAXgene tubes (1-2.5 ml, see 3.8.3.1) (paper II-III)
- 2 Cell Preparation Tubes (CPT) (16 ml, see 3.8.5.1) (paper IV)

3.8. Laboratory methods (paper I-IV)

3.8.1. Identification of mycobacteria (paper I-III)

Sputum and gastric aspirate smears were heat fixed, stained with Auramine O dye, destained using acid alcohol and counterstained with potassium permaganate before fluorescent microscopy was performed by trained personnel. For mycobacterial culture the same specimen were digested and decontaminated using the NALC-sodium hydroxide method, and inoculated onto Löwenstein Jensen medium (L-J) (300 µl) and Mycobacterial Growth Indicator Tube (MGIT) (500 µl). The cultures were followed for 8 weeks. Positive smears and cultures were confirmed by Ziehl-Neelsen staining and speciated using the HAIN kit (GenoType® MTBC, Ver1, Hain Life Sciences, Germany). Direct PCR (The COBAS® TaqMan® MTB Test, Roche 2007) was done on culture negative specimen for infants with CXR suggestive of TB. Positive cultures were tested for drug susceptibility (streptomycin, isoniazide, rifampicin, ethambutol).

3.8.2. QuantiFERON®- TB Gold In-Tube (paper I-IV)

The QuantiFERON®- TB Gold In-Tube (QFT) assay (Cellestis) was carried out according to the manufacturer's instructions. Briefly, venous blood was collected directly in the three kit tubes; Nil Control, TB Antigen and Mitogen control. Immediately after drawing of blood, the tubes were vigorously shaken before incubation at 37^{o}C for 16-24 hours. Plasma from the tubes was harvested before storage at -20 ºC, and transport in cryoshippers to the laboratory at the St- John's Research Institute, Bangalore, for the ELISA assay. As recommended by the manufacturer,[72] the standard curve for reading of the optical density (OD) values was based on 4 standards (S1-S4) prepared by dilution of the Kit standard. Triplicates of S1-S4 were analyzed in the middle of every 96-well plate which also contained a batch of 28 study samples (triplicates). OD values were read on an automated photometer. Every plate was assessed with regard to established quality criteria acquired by the QuantiFERON-TB Gold In-Tube Analysis Software:

- S1: mean OD ≥0.600
- Coefficient of variance (CV%) for S1 and S2 ≤15%
- S3 and S4: variation between replicates ≤0.040 OD from their mean.
- The correlation coefficient calculated from the mean absorbance (=OD) of S1-S4 must be ≥0.98.
- Zero Standard: mean OD ≤0.150

The test results were interpreted as positive, negative or indeterminate as recommended by the manufacturer (Table 7).

Table 7. Interpretation of QuantiFERON-TB Gold In-Tube results

Nil (IU/mL)	TB Antigen minus Nil (IU/mL)	Mitogen minus Nil (IU/mL)	QuantiFERON-TB (IU/mL)
≤8.0	<0.35	≥0.5	**Negative**
	≥0.35 and <25% of Nil value	≥0.5	
	≥0.35 and ≥25% of Nil value	Any	**Positive**
	<0.35	<0.5	**Indeterminate**
	≥0.35 and <25% of Nil value	<0.5	
>8.0	Any	Any	

Retesting at higher dilutions of samples with QFT result >10 IU/mL was not performed and is not required by the manufacturer.

Paper I-III (NCS)

Children referred to the CVW had blood drawn for QFT if capillary Hgb ≥10 g/dL.

Paper IV (ACS)

Initially, QFT was not included in the study protocol. A protocol change was made to include repeated QFT testing at study day 360 and day 720.

3.8.3. Dual color Reverse Transcriptase-Multiplex Ligation dependant Probe Amplification (dcRT-MLPA) (paper II and III)

As the amount of blood which can be obtained from infants is limited, we used a novel high-throughput technique developed and described by Joosten et al,[211] which requires only 130-150 ng of total RNA for analysis of a predefined panel of 49 genes consisting of four housekeeping genes, used as internal controls, and 45 genes identified by the partners in the Bill and Melinda Gates foundation Grand Challenge

project #6 consortium as differentially expressed during *M. tuberculosis* infection and/or disease in adults, by screening of different populations by qPCR and microarray. For each target sequence, two half-probes were designed and ligation of these probes is required to get the PCR product. This, together with placing the probe-ligation sites close to an exon boundary, minimizes detection of contaminating DNA fragments. Known splice variants and single-nucleotide polymorphisms were also taken into account in probe design. Validation of the assay as well as the threshold value, were established by serial dilutions of chemically synthesized oligonucleotide templates using a validation probe set of 8 genes. The detection threshold was 20 copies of any oligonucleotide, corresponding to log2 transformed peak area of ≥7.64. The results from the dcRT-MLPA assay proved highly comparable to Taqman real-time PCR. The inter-assay correlation was found to be excellent (0.998) when independent duplicate samples were compared. The variance in the assay was analyzed by setting the total variation to 100% and determining the contribution by 4 components; donor variation (the variation within a cohort of healthy individuals), collection variation (the variation within the same individual over time), tube variation (the variation between 5 separate PAXgene tubes collected from the same healthy donor) and assay variation (the variation between temporarily different RT-MLPA assays). The contribution to the variance by these components varied somewhat from gene to gene, but the predominant factor for all genes was the donor component, underlining the adequacy of the method to detect differences between subjects classified by disease state. The standard deviation for dcRT-MLPA patient samples is normally in the range 35-45% (personal communication by Marielle Haks, Leiden University Medical Center, Netherlands).

All required regents for the dcRT-MLPA assay were obtained from the Department of Infectious Diseases, Leiden Medical University, Leiden, The Netherlands:

- Probes: right-hand and left-hand 5’ phosphorylated half probes
- Primers: Reverse transcription gene target specific primers, FAM labelled MLPA primers, HEX labelled MAPH primers
- Positive control: synthetic template oligonucleotides (hybridization templates)
- Human Universal Reference RNA

3.8.3.1.RNA extraction

Total RNA was extracted from blood collected on the PAXgene Blood RNA Tubes using the 'PAXgene Blood RNA kit' with RNase free DNase on-column digestion (both PreAnalytiX, Hilden, Germany) according to the manufacturer's instructions. The total RNA concentration and purity (A260/280 nm ratio) were measured using a Nanodrop spectrophotometer (Thermoscientific, Wilmington, Delaware, U.S.A) and ranged between 0.4 - 24.5 µg (average 6.6 ± 4.85 µg). The RNA quality was further assessed by agarose gel electrophoresis.

3.8.3.2.The dcRT-MLPA assay

Samples with a concentration <50 ng/µl were concentrated at 45ºC using a speed vacuum concentrator (Eppendorf AG, Hamburg, Germany), before the transcription step. The dcRT-MLPA experiment protocol is described in detail by the inventors of the assay.[211] In brief, the assay includes the following steps: 1)reverse transcription, 2)hybridization of gene-specific probes, 3)ligation of half-probes, 4)PCR reaction and 5)fragment analysis. All samples for paper II and III were run in duplicates. The human reference RNA and syntetic oligonucleotide were included on each 96-well plate. Reference RNA served as positive control for reverse transcription, whereas the oligonucleotide was positive control for the PCR-reaction. The amplified PCR products were diluted 1:10 with nuclease-free H2O and added to a mixture of Hi-Di-Formamide with 400HD ROX size standard. The PCR fragments were analyzed on a 3730 capillary sequencer in Gene scan mode (Life Technologies, Carlsbad, California, USA).

3.8.3.3.Processing of dcRT-MLPA data

Data was analyzed using GeneMapper software version 4.0 (Life Technologies, Carlsbad, California, USA) according to the GeneMapper version 4.0 manual. The default peak detection settings were inspected and adjusted if necessary. The peak area (in arbitrary units) of duplicates were averaged, and amongst four reference genes included in the assay (ABR, B2M, GAPDH and GUSB), GAPDH showed the least variance. (GUSB or β-glucuronidase, is a lysosomal enzyme which could also play a role in the immune response towards TB[212]). All other genes where thus normalized against GAPDH using Microsoft Excel spreadsheet software and

subsequently log2 transformed as described by Joosten SA *et al.* Of the 45 genes, 7 genes had expression levels below the cut off value of 7.64 (peak area < 200 arbitrary units) and one gene, CD14, co-localized with a primer-dimer peak and was therefore omitted from further analysis.

3.8.4. Multiplex bead array – Bio-Plex assay (paper II and III)

For the Bio-Plex assay, we used supernatants from the QFT assay (the Nil and TB Antigen tubes). A pilot study was conducted using 52 samples randomly selected from the study subjects included in paper II: children with TB disease (11), MTB infected children (29) and uninfected controls (12). The samples were screened by the 'Human cytokines 27-plex' kit (Bio-RAD Laboratories Inc., Hercules, California, USA) according to the manufacturer's instructions and measured by fluorescent intensity (FI) by the Bio-Plex 200 System empowered with Luminex xMAP technology. With each assay set-up, dilutions of a multiple standard (S1-S8) containing all the 10 proteins were set-up in duplicates on each plate together with patient samples. Due to sample availability patient samples were run in single wells. Analyses comparing the three clinical groups revealed discrete differences in 5/27 biomarkers (IL-5, IL-13, IP-10, IFN-γ and TNF-α). These 5 biomarkers were selected for inclusion in a customized 10-plex kit together with 5 biomarkers (IL-2, IL-6, IL-8, IL-10 and MCP-1) added because of findings in previous TB biomarker studies.[213-215] All the samples (210) were then tested by the 10-plex kit and analyzed both in the setting of paper II and paper III. The cytokine/chemokine concentrations (pg/mL) in the unstimulated (Nil) and stimulated (TB Antigen) tubes were analyzed individually.

3.8.5. 9-color flowcytometric assay on PBMCs (paper IV)

3.8.5.1. Isolation, storage and transport of PBMCs

At the study field-laboratory at Emmaus Swiss Hospital, peripheral blood mononuclear cells (PBMC) were isolated from 16 ml peripheral blood collected on 2 CPT tubes per subject, according to the manufacturer's instructions. Briefly, within 2 hours of venipuncture the CPT tubes were inverted 8-10 times prior to centrifugation (25 min of 1750g at room temperature (RT)). Following aspiration of plasma, cell layers from both CPTs (same individual) were collected into a 15 ml centrifugation tube, before 2 repeated washings with RPMI 1640 (Gibco 11875 or Sigma R8758) with 1% Fetal Bovin Serum (FBS) (Gemcell 100-500 or Gibco 16000-044)) and

centrifugations (370 g for 10 min at RT). The cells were resuspended and frozen in -80ºC immediately after adding an equal amount of 80% FBS with 20% DMSO (Sigma D2650). After 12 hours at -80ºC, the samples were transferred to -152 ºC for typically a week, before transport and banking in to the Biorepository liquid N_2 freezers at St. John's Research Hospital. Selected samples were shipped simultaneously in liquid N_2 (MVE Vapor Shipper) to Oslo University Hospital, Norway, where the samples were randomized for processing in 7 batches, each containing a maximum of 24 samples.

3.8.5.2.Thawing

PBMC were thawed by drop-wise resuspension in preheated RPMI 1640 (BioWittakre) with 10% FBS (Sigma) and 1% L-glutamine (Lonza, BioWhittaker), and left over-night. Cell counts and viability were assessed on Tryphan Blue stained cells (Countess, Invitrogen), and in some samples confirmed by manual counts after staining with Acridin Orange (Sigma). Samples with cell viability >70% were set up for further analysis.

3.8.5.3.*In vitro* stimulation

PBMCs were assessed for *ex vivo* responses to peptide pools of 1) Ag85b (2 µg/mL), 2) Early Secretory Antigen Target-6 (ESAT-6) (2 µg/mL), 3) Rv 2660c (10 µg/mL) and 4) Purified Protein Derivate RT50 (PPD) (10 µg/mL), all provided by Statens Serum Institutt, Copenhagen, Denmark. Staphococcal Enterotoxin B (SEB) (Sigma) was used for positive control and PBS served as negative control, all in the presence of the co-stimulants αCD28 (10 µg/mL, clone L293, BD) and αCD49d (10 µg/mL, clone L25, BD). Samples from the same subject (QFT baseline and after 1 year) were set up side by side on a 96-well round bottom plate (Corning Life Sciences. Brefeldin A (BD GolgiPlug) (1.0 µl/well) and monensin (BD GolgiStop) (0.7 µl/well) were added before the 6 hour incubation (37ºC, 5% CO_2).

3.8.5.4.Simultaneous staining of membran and intracellular proteins

As CD107 (BD) is a degranulation marker, CD107a-FITC (BD) was added together with the Golgi inhibitors prior to incubation. Stimulated samples were washed in PBS, stained with LIVE/DEAD Violet Fixable Dead Cell Stain (L34955, Invitrogen), incubated for 10 min, washed with PBS, then stained for the following surface

markers; CD3-APC Alexa Fluor 750 (Beckman Coulter), CD4-PerCP Cy5.5 (BD), CD14-PacBlue (BD), CD16-PacBlue (BD), CD19-PacBlue (Invitrogen) and CD45RO-PE Cy5 (BD), and incubated for 25 min. The cells were subsequently washed twice with FACS buffer (PBS with 2% FBS and 0.01% sodium azide), fixed and permeabilized by a 10 min incubation using Cytofix/Cytoperm (BD) and subsequent wash with Perm/Wash Buffer (BD). Intracellular markers were added; IFNγ-Alexa Fluor 700 (Invitrogen), IL2-APC (BD), TNFα-PE Cy7 (BD), and perforin-PE (Diaclone), before a 30 min incubation followed by two washes with Perm/Wash Buffer. Stained samples were stored in 1% formaldehyde at 4°C until flowcytometric analysis (within 48 hours). All staining steps and incubations were done at room temperature.

3.8.5.5.Flowcytometric analysis

The samples were run on a LSR II flowcytometer (BD Biosciences) using the configuration and set-up given in Table 8.

Table 8. Configuration and set-up of the "Yellow LSRII", Montebello

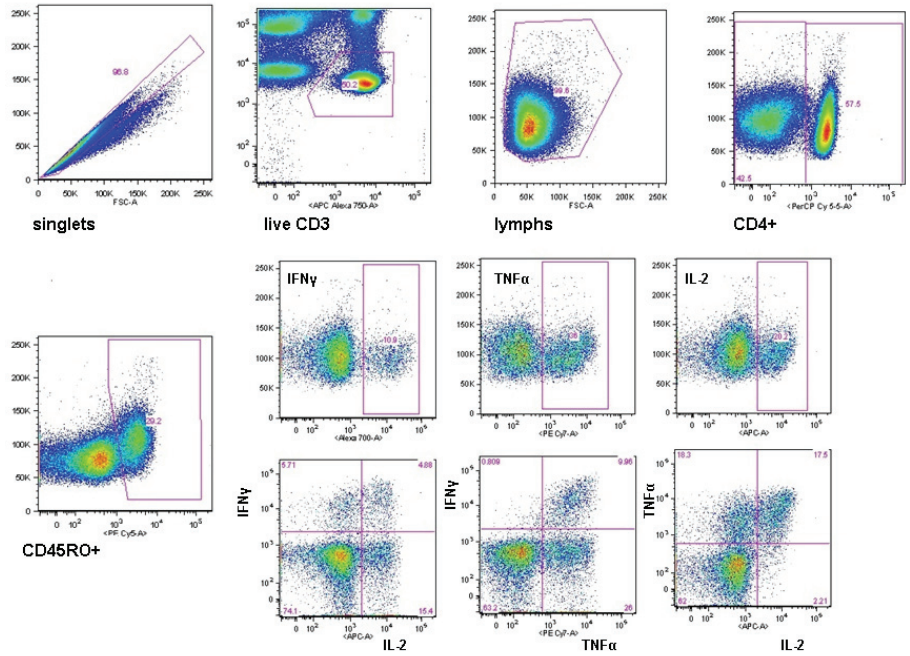
Laser	Marker	Flurochrome	Filter
Violet 405	Dump/viability	Pacific Blue	450/40
Blue 488	CD107a	FITC	525/50
	CD4	PerCP-Cy5.5	720/40
Yellow 561	Perforin	PE	582/15
	CD45RO	PE-Cy5	670/14
	TNFa	PE-Cy7	780/60
Red 640	IL2	APC	670/14
	CD3	APC-Alexa750	780/60
	IFNg	Alexa 700	730/45

To avoid high pressure in the high-throughput sample platform due to many fluorochromes, samples were transferred to FACS tubes, diluted in PBS and manually loaded. Single stained BD CompBeads were used for automatic compensation. For each individual sample, 20 000 events were recorded ungated for sample quality assessment, before the storage gate was set to a wide lymphocyte gate based on forward- and side scatter properties. FACS Diva Software (BD) was used for data acquisition.

3.8.5.6.Gating strategy and data extraction

After transfer of data to FlowJo Software, version 9.4.11, the compensation matrices were manually inspected an adjusted if appropriate. The expression of cytokines after specific short-time (18h) stimulation is reported very low within the CD45RO^{low} subsets,[216] thus we gated on the CD3+CD4+CD45RO+ and CD3+CD4-CD45RO+ cell populations followed by cytokine gates. The gating strategy is given in Figure 11.

Figure 11. Example of a representativ sample after stimulation with positive control (SEB). Singlets were gated based on forward- and side scatter properties; CD3 high cells not within the dump gate (CD14, CD16, CD19); verification of the lymphocyte population; CD4 high population; CD45RO high (memory) population; single cytokine gates (upper pane) and two-dimentional cytokine gates (lower pane) were used to correctly draw the gates according to the "natural break".



Boolean gates were created based on combinations of single cytokine gates. Both a cytokine panel (IFNγ, IL-2, TNFα) and a cytotoxic panel (CD107a, IFNγ, perforin) was analyzed; the cytotoxic panel was assessed within CD3+CD4-CD45RO+ cells only. Samples excluded from data analysis (Figure 12) had either

- a poorly defined lymphocyte population,
- CD3+CD4+ or CD3+CD4- cell counts <5000 or
- lacked a valid positive control. (Valid positive control defined as >1% cytokine-producing cells when stimulated with SEB.)

When assessing cytokine-positive cells, the background response to PBS was subtracted. A threshold for positive cytokine response was set at a 2-fold increase in cell count above the background, and samples that did not meet this requirement were set to zero. Subjects producing any of the cytokines were considered responders to the respective antigens. About 50% of the consistent positives responded to ESAT-6 and Ag85b, but as the frequencies of cells responding to Ag85b were considerably lower, data on Ag85b-responses was not included in paper IV. Neither was data on Rv2660c-responses as only 25% of the consistent positives responded to this antigen.

3.9. Statistical analysis

All p-values were calculated using two-tailed tests and a p-value of <0.05 was taken to be significant. When not stated otherwise, Microsoft Office Excel 2003 were used for data handling, analyses were conducted using the PASW Statistics version 18.0 and GraphPad Prism version 5 was used for statistical figures.

Paper I

Sociodemographic, clinical and nutritional characteristics were assessed in the 705 children classified according to the diagnostic TB algorithm, as follows: Low birthweight (<2500g) and being small for gestational age (SGA: Weight-for-gestational age $<10^{th}$ percentile)[217] were used as indicators of intra-uterine nutritional status. Birthweight was also assessed as a continuous variable. The WHO child growth standard Z-scores were dichotomized using the cut-off <-2, which defines wasting (weight-for-height (WHZ)), stunting (height-for-age (HAZ)) and underweight (weight-for-age (WAZ) and BMI-for-age (BAZ)).[209] Equal to the WHO definition, hemoglobin <10 g/100ml defined the cut-off for moderate-severe anemia.[218] Age was categorized into 3 levels (<12 months, 12-23 months, ≥24 months and also assessed as a continuous variable. Data on mother's and father's education were captured in 6 levels, but recoded into three categories for data analysis (*illiterate, primary or secondary school, high school or higher*). Housing material and cooking fuel were dichotomized in order to identify higher socioeconomic status (*bricks* versus *others; electricity or gas* versus *others*, respectively). TST and QFT were handled as categorical variables; TST dichotomized around the cut-off ≥10mm as recommended in local guidelines,[210] and QFT was divided into 3 categories as recommended by the

manufacturer: positive (≥0.35 IU/mL), negative (<0.35 IU/mL) and indeterminate.[72] For QFT, exact measurements >10 IU/mL were not available, due to the technical restriction imposed by the test's standard curve. Differences in the distribution of the variables between children with clinical TB and children without TB were assessed: Continuous variables by T-test (normally distributed) or Mann Whitney U test (not normally distributed); Categorical data by Pearson's chi-squared with Yates Continuity Correction or Fisher's exact test, where appropriate.

Kappa statistic (κ) was used in the concordance analyses of TST and QFT results for individuals with valid results for both tests, and evaluated in accordance with McGinn et al..[219] Spearman's Rank Order Correlation was used to assess the relationship between the mitogen response and age. (The result from this analysis is not included in paper I, but in section 5.2).

Because we observed a considerable left-skewing in the distribution of the nutritional Z-scores, the nutritional variables WHZ, HAZ, WAZ and BAZ were reassessed to optimize the definition of these variables before inclusion in univariate and multivariate logistic regression analyses. The Z-score variables were analyzed in different ways: 1) as continuous variables, 2) as categorical variables defined by the standard WHO cut-offs; <-2 (moderate to severe malnutrition); <-3 (severe malnutrition), 3) after re-standardization of the cut-off (mean minus 1.96*St.dev), 4) binned by quartiles and 5) dichotomized around cut-offs corresponding to the lowest quartiles (new cut-offs: WHZ <-2.61; HAZ <-2.16; WAZ <-2.65; BAZ <-2.51). Variables which turned out significant when continuous did not do so when categorized as in alternative 2) and 3). Based on this, we concluded that dichotomous variables with a cut-off corresponding to the lowest quartile, best reflected the information in the dataset. The left-skewing in Z-score distributions and new cut-offs used in logistic regression analyses are illustrated in Figure 12.

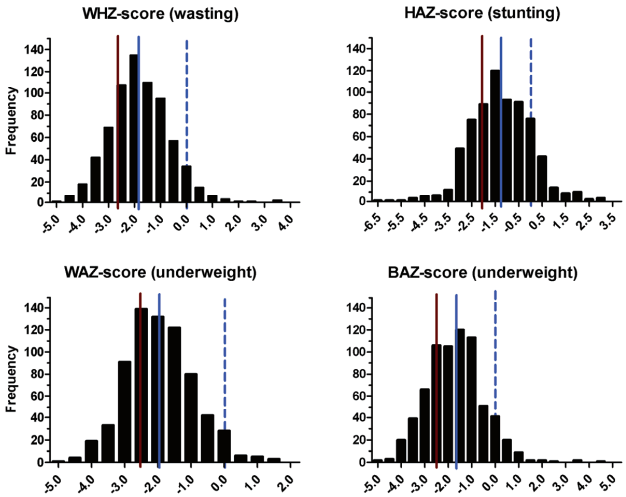


Figure 12. Left-skewed WHO Z-scores in the study population. In each plot, the mean Z-score in the study population is marked with a blue line compared to the WHO Z-score means (dashed blue line). New cut-offs corresponding to the lowest quartile is marked with a red line.

Associations with the following outcomes were assessed by univariate and multivariate logistic regression analyses:

1. a positive TST versus a negative TST
2. a positive QFT versus a negative QFT
3. concordant TST and QFT positive versus concordant negative or discordant positive for TST and QFT.
4. an indeterminate QFT versus a valid QFT result (positive or negative)

Unadjusted odds ratio estimates for the variables: surveillance arm (active or passive), gender, birthweight, SGA, age, socioeconomic factors (mothers/fathers education, housing, cooking fuel), known TB exposure, symptoms, nutritional status (WHZ, HAZ, WAZ, BAZ, anemia) and isolated NTMs, were performed by logistic regression.

Variables with significant univariate associations with the TST and/or QFT outcomes were considered for inclusion in the multivariate model if they, based on the literature, could be assumed to have a casual impact on the outcomes. These assumed causal relationships were based on the literature and drawn by principles of a directed

acyclic graph (DAG).[220] Notably, as the positive TST and/or QFT outcomes are imperfect read-outs of the clinical TB outcome (clinical TB disease, MTB infection or no infection/disease) for which there is no gold standard, we chose to illustrate the variables relationship both to the clinical TB outcome and the positive TST and/or QFT outcomes (Figure 13) (Causal relationships are indicated with arrows.) The variables' impact on clinical TB influence on the child's susceptibility for MTB infection and disease, whereas the variables impact on a positive TST and/or QFT result influence on the ability of the tests to correctly reflect the clinical TB outcome (test performance). Following the DAG principle, ancestor variables (variables that is a cause of another variable more closely linked to the outcome) and descendants of the outcome, were not included in the multivariate models.[220] These considerations left age, TB exposure, WHZ, HAZ and NTM presence in the multivariate model together with gender which is routinely included. WHZ and HAZ represent different entities of malnutrition, and important colinearity or interaction was not found when all possible 2-way interactions in each of the multivariate models were tested. Nevertheless, their inter-relationship both as continuous and categorical variables was investigated with regards to correlation (Spearman's rho=-0.06, p=0.14), colinearity (Tolerance=1, Variance Inflation Factor=1) and interaction. Analyses suggested a higher correlation between weight-for-age Z-score (WAZ) and either of WHZ (Spearman's rho=0.49, $p<0.0005$) or HAZ (Spearman's rho=0.37, $p<0.0005$). A slight interaction between WHZ and HAZ ($p=0.048$) for a positive QFT was found. The model including the interaction gave a better fit (Likelihood Ratio test Statistics: $X^2=5.34$, $p<0.025$), but did not change the coefficients.

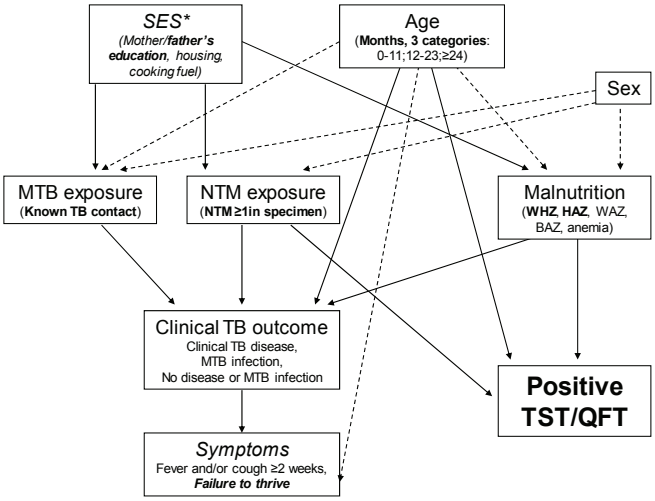


Figure 13. A Directed Acyclic Graph (DAG) illustrating the causal relationships between the determinants for the outcomes clinical TB and positive TST and/or QFT. A causal relationship between a determinate (variable names listed in parenthesis) and an outcome is indicated with arrows. Ancestors (variables that is a cause of another variable more closely linked to the outcome) and descendants of the outcome not included in the multivariate models, are in italic. Variables significantly associated to the outcomes in univariate analysis are in bold. Dashed arrows indicate more uncertain relationships.

Regarding the indeterminate QFT outcome, the assumed causal relationship is different (Figure 2). Being primarily the result of inadequate mitogen responses, this outcome is not specific for clinical TB outcome, but rather affected by all physiological conditions interfering with T-cell responses in general. Accordingly, known TB was not considered relevant whereas symptoms of any duration (categories defined in Table 1, paper I) were included because of previous reports.[221] Notably, age was used as a continuous variable in the multivariate model as categorization gave statistical problems due to no indeterminate results in the oldest age group. As inclusion of variables which measure the same thing can cause statistical problems related to co-linearity, we chose to exclude the symptom FTT and instead use the Z-score to facilitate the interpretation in relation to the positive TST and/or QFT model. In addition, FTT in our study was a composite variable including 3 different aspects related to the weight-for-age Z-score. Applying "clean" Z-scores makes comparison with other studies easier (Figure 14).

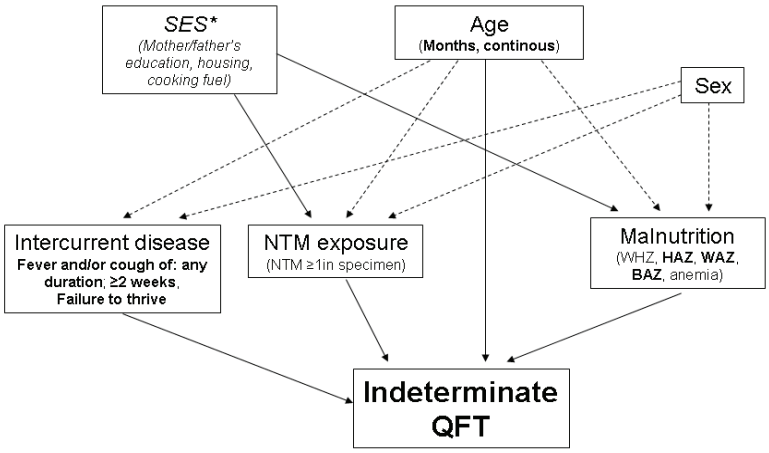


Figure 14. A Directed Acyclic Graph (DAG) illustrating the causal relationships between the determinants for the outcome indeterminate QFT. A causal relationship between a determinate (variable names listed in parenthesis) and an outcome is indicated with arrows. Ancestors (variables that is a cause of another variable more closely linked to the outcome) and descendants of the outcome not included in the multivariate models, are in italic. Variables significantly associated to the outcomes in univariate analysis are in bold. Dashed arrows indicate more uncertain relationships.

As WAZ and BAZ are alternative markers of nutritional status and indicate underweight if the Z-score is <-2),[222] exchanging WHZ and HAZ for WAZ or BAZ in the multivariate models was also explored.

The amount of variation explained by the final multivariate models (Nagelkerke R Square) for the different TST/QFT outcomes is given in (Table 9).

Table 9. Explained variation (Nagelkerke R^2) by the multivariate model

Outcome analyzed	R^2
TST positive (≥10mm)	0.385
QFT positive (≥0.35IU/mL)	0.102
Combined TST/QFT positive	0.267
Indeterminate QFT	0.193

Age was the most important factor and contributed with 60-80% of the explained variation in all models, whereas sex and NTM presence did not contribute in any model. Known TB contact contributed more to the explained variation for the

outcomes *QFT positive* and *Concordant TST/QFT positive* (both ~20%) than for *TST positive* (~4%). The contribution by nutritional variables (WAZ for *TST positive* and HAZ for *QFT positive* and *Concordant TST/QFT positive)* were as important as known TB contact in all these models. For the outcome *Indeterminate QFT*, symptoms contributed more than the nutritional variables, but overlap between these variables is likely as 3 different models including sex, age and either 1)symptoms, 2)WHZ or 3)HAZ all explained ~80% of the explained variation in the final model.

Paper II

Differences in baseline characteristics were identified by the Pearson's chi-squared test (with Yates continuity correction) or Fisher's exact test, where appropriate. dcRT-MLPA (gene expression) and bio-plex (cytokines/chemokines) data were analysed separately following the analysis strategies given below:

1. The global test was performed to determine whether the global expression pattern of the selected genes was related to any of the clinical outcomes (pair-wise comparison).[223]

2. Binary logistic regression was applied for the identification of single biomarkers differentially expressed between the clinical groups. These analyses were performed with and without adjustments for age (months), nutritional status (WHZ and WAZ) and NTM in specimen (categorical variables as given in Table 1, paper II). These factors were regarded possible confounders based on their differential distribution between the clinical groups. Later, the adequacy of adjusting for these factors was re-evaluated by the principles of a DAG diagram (defined and described in section 3.9, paper I) (Figure 15), and confirmed. No correction for multiple testing was undertaken due to the exploratory nature of the study.

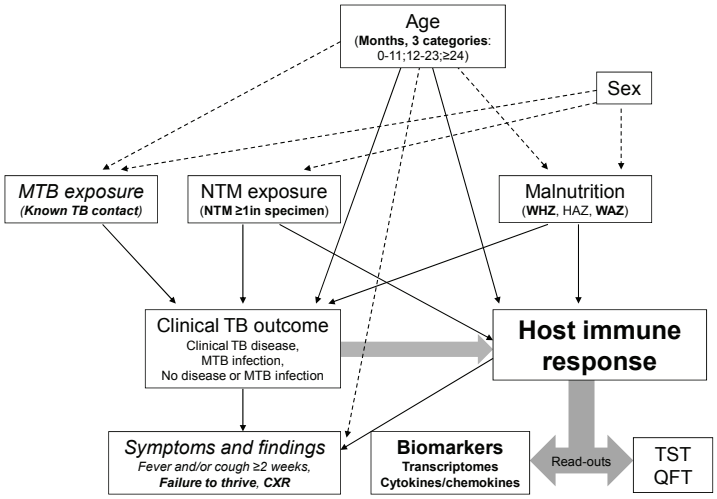


Figure 15. A Directed Acyclic Graph (DAG) illustrating the assumed causal relationships between the determinants for clinical TB outcomes and host immune response for which biomarkers, TST and QFT were the available read-outs in our setting. A causal relationship between a determinate (variable names listed in parenthesis) and an outcome is indicated with arrows. Dashed arrows indicate more uncertain relationships. Variables significantly associated with the clinical TB outcome in univariate analysis was adjusted for when assessing differences in biomarker profiles between the clinical TB outcome groups are in bold. Ancestors (variables that is a cause of another variable more closely linked to the outcome) and descendants of the outcome not adjusted for, are in italic.

3. The "lasso" (Least Absolute Shrinkage and Selection Operator) is a regression model which penalizes the absolute size of the regression coefficients, shrinking them towards zero. This can be an appropriate approach if the predictors are likely to be highly correlated or interdependent. In either case, standard regression may generate overly-generous regression coefficients 51. It is an alternative to backwards stepwise elimination of non-significant terms, not relying on statistical significance tests. As a result, lasso is useful for establishing a prediction model at an individual level (i.e., identifying important predictors), but not for summarizing average trends in the data.[224] The optimal lasso model is the one with optimal lambda value or penalty. For the biomarker data, this was determined using leave-one out cross-validation. This method replaces the classical adjustment for multiple testing.[224] The lasso model corresponding to the lambda value which resulted in the smallest

prediction error was considered the optimal model and the non-zero estimated coefficients were reported. Next, the prediction performance of this model in terms of probabilities and ROC curves, was based on the performance on the test data

The statistical environment R was used for the global test[223] and for the lasso regression (add-on package "glmnet").[225]

Paper III

Differences in baseline characteristics and single biomarkers between subjects classified according to TST and/or QFT results were analyzed as described for paper II. Biomarker signatures using the global test and/ or lasso regression were not considered relevant in this setting.

Paper IV

The distributions of age, sex and BCG-status between the QFT categories were assessed by ANOVA and Fisher's Exact test where appropriate. For TST, QFT and absolute frequencies of T-cells subsets (flowcytometric data), non-parametric statistics were applied as the data were not normally distributed. As the flowcytometric data on relative frequencies are normalized, parametric statistics (unpaired T-test with Welch's correction) were applied.[226] The study was designed to have paired measurements for all immunological read-outs. For TST and QFT data, paired analyses on repeated measurements by the Wilcoxon Signed rank test was performed, whereas differences between groups (unpaired data) were assessed by Mann-Whitney U test. High quality demands for flowcytometric samples resulted in too many missed samples (section 3.8.5.6) to allow for paired statistical analyses (Friedman's test). Therefore, pair-wise comparisons by the Mann-Whitney U test were done. Correlations were assessed by Spearman's Rank Order Correlation. A two-tailed α-level of $p<0.05$ was considered significant. Microsoft Office Excel 2003 and GraphPad Prism version 5 were used for data handling and statistical analyses.

3.10. Ethical considerations

The NCS and ACS were conducted according to the principles of the Declaration of Helsinki (4th revision), and approved by the institutional review board at St. John's National Academy of Health Sciences, and an independent ethics review committee

contracted by the Aeras and the Ministry of Health Screening Committee, Government of India.

- NCS: No. 5/8/9/60/20006-ECD-1 dt.10.11.2006
- ACS: No. 5/8/9/52/2006-ECD-I dt. 10.11.2006

Confidentiality of patient identify was maintained by assigning each subject with a coded number used to identify all documents and specimens. All source documents are stored in a secured location, and except for monitoring and auditing of the study, a written assent is required for any dissemination. The informed consent process for the NCS is described an evaluated in detail by Rajaraman et al.[207]

4. MAIN RESULTS – summary of papers

4.1. Paper I

Influence of Age and Nutritional Status on the Performance of the Tuberculin Skin Test and QuantiFERON®-TB Gold In-Tube in Young Children Evaluated for Tuberculosis in Southern India

Jenum S, Mahelai D, Selvan S, Jesuraj N, Vicky Cardenas, Kenneth J, Hesseling AC, M Pai, Doherty TM , Vaz M, Grewal HMS, TB Trials Study Group.

A reliable identification of young children with *Mycobacterium tuberculosis* (MTB) infection or tuberculosis (TB) is vital to assure adequate preventive and curative treatment. As pediatric TB often remains unconfirmed, evidence of a previous encounter with MTB by a positive Tuberculosis Skin Test (TST) or IFNγ-release assay (IGRA) are used as a supplement in the diagnosis, but data on the performance of these tests are scarce in children aged <5 years. Whereas an increasing number of studies have addressed how immune suppression in the context of HIV infection affects the performance of TST and IGRAs in young children, the impact of immune suppression by malnutrition is far less studied. Therefore, our aims were to evaluate the performance of TST and QuantiFERON-TB Gold In-Tube (QFT) and their association with clinical-, demographic- and nutritional (intra-uterine and post-natal) characteristics in a cohort of children aged <3 years in a setting with high TB burden and low HIV prevalence.

Nested within a prospective cohort study of 4382 neonates in Southern India, this study included 705 children aged 1-35 months (mean 14.8) evaluated for TB during the 2-year follow-up. 13 children were diagnosed with clinical TB, in whom MTB was confirmed in 4. Isolated isoniazid resistance was detected in 1 out of 3 children with culture confirmed TB. The sensitivities of TST and QFT for clinical TB were equally poor (31% and 23%, respectively). Based on adjusted logistic regression of TST and QFT outcomes, the poor sensitivity of these tests in this population seems, at least in part, attributable to young age and malnutrition. More specifically, children aged <2 years were less likely to have a positive TST and/or positive QFT and more likely to have an indeterminate QFT. Children within the lowest quartile for the height-for-age Z-score, indicative of chronic malnutrition, were more susceptible to MTB infection and more prone to indeterminate QFT results. TST was less reliable in children with a weight-for-height Z-score within the lowest quartile.

4.2. Paper II

Identification of biomarkers for Mycobacterium tuberculosis infection and disease in BCG-vaccinated young children in Southern India.

S. Dhanasekaran, Synne Jenum, Ruth Stavrum, Christian Ritz, Daniel Faurholt-Jepsen, John Kenneth, Mario Vaz, Harleen M. S. Grewal, T. Mark Doherty, TB Trials Study Group **(Genes Immun 2013;May 16)**

There is an urgent need for better diagnostic tools in the diagnosis of MTB infection and disease in young children. Our aim was to explore biomarkers with a potential to discriminate between clinical TB (n=13), MTB infection (n=90) and neither (controls , n=107) in children <3 years with suspected TB. mRNA isolated from unstimulated peripheral blood mononuclear cells was examined for the transcription of 45 genes. A 10-plex assay was applied to analyze the level of cytokines/chemokines in QFT supernatants (TB Antigen and Nil tube). The clinical groups were compared pair-wise with and without adjustments for age, nutritional status and the presence of non-tuberculosis mycobacteria, both with regard to single gene expression (logistic regression) and with regard to biosignatures (Global test and lasso regression).

In adjusted analyses of single gene transcripts in unstimulated peripheral blood, we found lower levels of *RAB33A* in clinical TB compared with MTB infection and controls, whereas mRNA for *SEC14L1, GUSB, BPI, CCR7*, and *TGFB1* were reduced in TB patients compared to MTB infected children or controls. Children with MTB infection displayed higher levels of *CD4, TGFB1* compared to controls. Lasso regression was applied to identify the biomarker signatures which best predicted the clinical outcomes. *RAB33A* alone discriminated between clinical TB and MTB infection (AUC 77.5 %), whereas a biosignature of *RAB33A, CXCL10, SEC14L1, FOXP3,* and *TNFRSF1A* effectively discriminated between clinical TB and controls (AUC 91.7 %). This supports, also in young children, the diagnostic potential of *RAB33A* for clinical TB and in the discrimination between MTB infection and disease. The 5-biomarker signature and *CD4* and *TGFβ-1* might provide clues to the identification of clinical TB cases among children with symptoms for other reasons. The 10-plex cytokine assay on TB Antigen supernatants revealed little difference between the clinical groups, but increased levels of IL-2 and IL-13 was found in children with MTB infection compared to controls.

4.3. Paper III

Concordant or discordant results by the tuberculin skin test and the QuantiFERON-TB test in children reflect immune biomarker profiles.

S. Dhanasekaran, Synne Jenum, Ruth Stavrum, Christian Ritz, John Kenneth, Mario Vaz, T. Mark Doherty, Harleen M. S. Grewal, TB Trials Study Group

Young children have an increased risk of progression to TB following MTB infection. Therefore, evidence of MTB infection, as indicated by a positive TST and/or positive QFT, should initiate preventive treatment in children aged <3 years when active TB has been excluded. However, TST and QFT often give discordant results in this population. How to interpret discordant results in terms of TB risk and preventive treatment is unclear. We hypothesized that a preselected panel of TB immune biomarkers could provide some insight into the immunological mechanisms underlying concordant and discordant TST and QFT results. Children were classified according to TST and/or QFT result and the expression of 45 gene transcripts in peripheral blood (*direct ex vivo*) and 10 cytokines/chemokines in QFT supernatants (Nil and TBAntigen tubes from the QFT assay) within these groups, were compared pair-wise by logistic regression, with and without adjustments for age, nutritional status and the presence of non-tuberculous mycobacteria.

Comparing test positive children to concordant negative (TST-/QFT-) children, concordant positive (TST+/QFT+) children only, had differentially expressed transcriptomes of *FPR1, TNFRSF1A* (both up-regulated) and *BPI* (down-regulated), and increased levels of IFNγ (TB-Ag tube). Contrary, all test positive children (TST and/or QFT positive) had up-regulation of the transcriptomes *CD4* and *TGFB1*, and increased levels of the cytokines IL-2 and IL-13. The only difference between the TST+/QFT- and TST-QFT+ discordant children was a down-regulated transcription of *SEC14L1* and an elevated level of IP-10 in TST-/QFT+. IP-10 proved to be more differentially expressed between concordant/discordant groups than IFNγ, and seemed in fact to be a better marker for a positive QFT.

4.4. Paper IV

The frequencies of IFNγ+IL2+TNFα+ PPD-specific CD4+ memory T-cells correlate with the magnitude of the QuantiFERON® Gold In-tube response in a prospective study of healthy Indian adolescents.

Jenum S, Grewal HMS, Hokey D, Kenneth J, Vaz M, Doherty M, Jahnsen FL, TB Trials Study Group.

MTB infected subjects who do not progress to tuberculosis (TB) are assumed to possess natural protective immunity. Their immune responses have been extensively studied to identify immune correlates of protection from TB progression, highly needed for TB vaccine trials and more targeted preventive treatment. The QuantiFERON® Gold In-tube assay is an IFNγ-release assays used to identify MTB infected subjects. Evidence suggests a positive correlation between the magnitude of specific IFNγ-responses and TB risk, but studies on the single cell level suggest that the quality (polyfunctionality) of the T-cell response is more important for protection than the quantity of IFNγ-release. This assumption has not been supported by prospective, longitudinal studies. We hypothesized that the quality and magnitude of MTB-specific T-cell responses in peripheral blood would differ between two groups of healthy subjects with different TB risk, as judged by their longitudinal QuantiFERON®-TB Gold In-Tube (QFT) results. As part of a prospective cohort study in Indian adolescents, we assess, by multicolour flow cytometry, the cytokine production by mycobacterial-specific T cells in PBMCs of 42 QFT positive adolescents of whom 21 became QFT negative (reverters) within 1 year. Ten QFT consistent negatives were included as controls.

Relative frequencies of PPD-specific polyfunctional (IFNγ+IL2+TNFα+) CD4+ T-cells were high but similar in both QFT groups at baseline. Reverters displayed lower absolute frequencies of these cells, which were further reduced 1 year later. Notably, the polyfunctional subset was most efficient in terms of cytokine production and their absolute frequency correlated well with the magnitude of the QFT-response. Our data do not support that higher relative or absolute frequencies of PPD-specific polyfunctional CD4+ T-cells in peripheral blood can explain the reduced risk of TB progression observed in QFT reverters. Contrary, absolute frequency of these cells correlated with the QFT-response, suggesting that this readout reflects antigenic load.

5. GENERAL DISCUSSION

5.1. Discussion of methods

5.1.1. Study design of NCS and ACS

The strength of these studies is the prospective design, the considerable number of enrolled subjects and the population based setting in a low- to middle income country with low prevalence of HIV. This design was required to establish the true incidence of TB in children <3 years and adolescents in order to prepare the study area for future vaccine trials. The study design and data acquisition within NCS and ACS constitutes a fixed framework with possibilities and limitations when it comes to answering other relevant research questions in the field of TB research. Nested within NCS and ACS, the study designs in paper I-IV were chosen to make the results and findings transferable and relevant to clinical decision making in the diagnosis and treatment of MTB infection and disease. Paper I, III and IV shed light on current diagnostic tools, whereas potential future diagnostics and predictive markers are explored in paper II and IV.

A limitation by the NCS/ACS design is the per protocol pre-scheduled follow-up assessments. This implies that the time since TB exposure is likely to be more heterogeneous than in household contact studies. The accuracy of exposure data is likely to decrease with the time elapsed from the encounter with an index case, and the pre-scheduled follow-up. The heterogeneity of exposure intensity will also be broader in a population-based versus a household contact setting. Nevertheless, the kinetics of the host immune response read-outs included in the papers (TST, QFT, biomarkers and MTB-specific T cell responses) are largely unknown,[30, 227] and is practically unachievable to establish in humans. Subsequently, optimized timing for testing is difficult to assure in study protocols.

Paper I-III

It is a strength that the study setting resembles a “real-life” context where parents present their ill child for diagnosis and treatment. This makes the conclusions clinically relevant. The proportion of children with malnutrition and the high

background presence of NTMs may limit the generalizability to other settings, but these conditions are typical for many areas with high-to-moderate TB burden.

Ideally, data on the gradient of TB exposure should have been recorded. As a gold standard for MTB infection is lacking, data on the exposure gradient and/or the infectivity of the index case is often used as a surrogate of MTB infection. If the magnitude of TST or IGRA results corresponds to the gradient of exposure, interpretation and validation of these diagnostics would be facilitated.[62, 63, 228-233] Many argue that TB exposure is a better surrogate of MTB infection in TST/IGRA validation than confirmed cases of active TB, as active disease by itself likely affects the host immune response and the performance of immunological tests.[62]

Furthermore, data on helminth infection were not available. Co-infection with helminths influences host immunity[90, 234] resulting in increased susceptibility to MTB infection.[235] In addition, the likelihood of an indeterminate QFT seems to be increased.[89] The prevalence of helminth infection in the NCS study population is presumably high as a similar but older Indian population had a prevalence of 35%.[236] Acquisition of data on helminth infection is laborious and requires trained personnel. Therefore, in the context of NCS, we were unable to collect stool samples.

Paper IV

In the context of MTB infection and disease, repeated data on the cytokine profile of specific T cell subsets are limited to settings related to treatment response. To our knowledge, polyfunctional mycobacterial-specific T cell responses have not been assessed in healthy subjects repeatedly tested by QFT outside a contact investigation setting. Furthermore, by assessing groups with different risk of TB progression as judged by repeated QFT results, our study design had the potential to provide polyfunctional T cell data relevant to TB risk assessment.

5.1.2. Definition of clinical outcomes

5.1.2.1. Clinical TB (paper I and II)

In real-life, clinical TB cases comprise both cases confirmed by the finding of MTB in culture or by PCR (definite TB), and bacteriologically unconfirmed cases (probable/possible TB).[12] The validation of diagnostic tools requires a gold standard,

which in the setting of TB, can only be provided by confirmed TB cases. This is why the performance of TST and IGRAs in the diagnosis of MTB infection have been evaluated using confirmed TB cases.[61] At the same time, it is broadly accepted, also in adults, that a proportion of TB cases are smear/culture negative,[74, 237] thus these cases are systematically left out of evaluations on TST and IGRA performance. In young children, TB remains unconfirmed in 70-80%.[75-77] Therefore, the strict requirement for confirmed TB cases in the evaluation of diagnostic tests is rarely applied in studies of pediatric TB. Instead, clinical TB is the commonly applied outcome.[63] Whereas confirmed (definite) TB is clearly defined, the criteria for bacteriologically unconfirmed TB cases vary across studies[238-243] with consequences for the number of incident TB cases reported.[244]

The diagnosis of TB in this study relied on an *a priori* diagnostic algorithm (see figure section 3.3.1) with hierarchical classification of clinical TB outcome, as this approach seems to be most accurate in the assessment of incident TB cases.[244] Clinical TB was present in 13 of 746 (1.7 %) referred children. Despite throughout evaluation at the CVW, only 4 children (31%) had their diagnosis of active TB confirmed by MTB in culture or PCR, but this proportion is similar to other studies. [75] 9 children were classified as probable TB (see section 3.3.1, Figure 9). The diagnostic algorithm did not include 1) lack of response to broad-spectrum antibiotics (not fluoroquinolones) as suggested in some practical guidelines to reduce over-diagnosis of bacteriologically unconfirmed TB cases,[74] or 2) response to TB therapy used by others.[245] The definition of probable TB in NCS relied on the approach used by South African Tuberculosis Vaccine Initiative (SATVI), which have conducted multiple large-scale studies on TB incidence and vaccine trials (http://www.satvi.uct.ac.za/). The estimated incidence of TB in South Africa is 993 per 100 000[52] and probably even higher in densely populated Cape Town which constitutes the field for SATVI. This is about 5-fold the incidence of TB in India (176 per 100 000 population per year).[246] Subsequently, the same diagnostic algorithm is likely to produce more false positives in our setting. On the other hand, both the diagnostic algorithm and the diagnostic procedures focused on pulmonary TB with a subsequent risk of under-diagnosing extra-pulmonary TB. Whereas all children were palpated for enlarged cervical lymph nodes, extensive and often invasive investigations might be required in the diagnosis of other forms of extra-pulmonary TB. This was not done in NCS, and

would hardly be ethical on study participants without reasonable indications for extra-pulmonary manifestations. In South African children, extra-pulmonary TB were reported in 8.2% of non-HIV-infected children aged <3 years. Cervical lymphadenitis and TB meningitis accounted for 84%. Miliary TB was defined as intra-thoracic TB and occurred in 3.9%.[26] Children with severe manifestations like miliary TB and TB meningitis are severely ill and therefore likely to have been brought in for health care in an area where the consciousness of TB was high due to increased awareness caused by the on-going study. Osteoarticular TB in children is most likely to occur 1-3 years after the primary infection[22] and is therefore rare in young children.[26]

Children assessed at the CVW had a mean follow-up time of 8.5 months (range 0-22 months) after discharge, and 85% (paper I) and 77% (paper II) had active surveillance (visited by study staff bimonthly). 39 children came for repeated evaluations at the CVW without being diagnosed with TB. The mean follow-up time and large proportion of children with active surveillance make it unlikely that severe TB cases were missed. Notably, 5 children died after dismissal from the CVW. Verbal autopsy (questionnaire modified from an earlier published Indian study[247]), was performed for all these children (Table 10) without TB being considered a plausible cause of death. Nevertheless, these children could theoretically represent lost TB cases.

Table 10. Causes of death according to verbal autopsy in referred children.

Sex	Diagnosis		Data from the CVW					
	ICPC	Description	Time between admission and death	Age (mo.)	Results from TB assessments	WHZ	HAZ	WAZ
girl	N17	Acute Renal failure	1 month	2	Cough 2 weeks. CXR/TST/QFT negative. MOTT (indeterminate) in 1/2 induced sputum samples.	-3.68	-1.02	-3.30
girl	A09	Infectious gastroenteritis	9 months	3	Culture/CXR/TST/QFT negative.	0.27	-2.56	-2.05
girl	J22	Unspecified acute lower respiratory tract infection	3 weeks	4	Fever 2 months, cough 2 weeks. Culture/CXR/TST/QFT negative.	-4.12	-6.79	-7.16
boy			17 months	6	Culture/CXR/TST/QFT negative. Exposed to TB case on treatment	-2.62	-1.00	-2.51
girl			3 1/2 months	12	TST 17mm. Culture/CXR/QFT negative.	-0.43	-4.55	-3.10

In exploring and evaluating potential biomarkers in TB diagnosis (paper II), all TB cases should ideally be confirmed by the identification of MTB in specimen. Even though there will always be uncertainty about the correctness of a bacteriologically unconfirmed TB diagnosis, it seems unreasonable to omit unconfirmed TB cases in

exploratory studies in pediatric settings. This group is after all the most difficult when it comes to decision to treat. Ideally, confirmed and unconfirmed cases should be explored as separate groups, but little can be done about the small number of smear/culture positive TB cases in the NCS. Therefore, we decided to club smear/sputum confirmed (definite TB) and bacteriologically unconfirmed (probable TB) TB cases when exploring biomarkers in paper II. We acknowledge that this is likely to have reduced the discriminatory power of single biomarkers and biomarker profiles.

5.1.2.2. MTB infection (latent TB) and uninfected controls (paper II)

In paper II children positive for TST and/or QFT but without CXR changes consistent with TB, were categorized as latent TB. The term used for these children should preferably have been *MTB infection without overt disease*. Calcification of TB lesions is generally regarded as an indication of clinical quiescence or latency, which usually occurs 12-24 months following primary infection (even though it might come earlier in young children).[22] All children in this study are <3 years, and subsequently, the large majority of infected children can be assumed to be in the course of primary infection and unlikely to have reached a stage of latency.

A positive TST and/or QFT were used in the classification of MTB infected children. As discussed in 1.1.6.1, neither tests can be validated due to the lack of a gold standard. Furthermore, the tests might give discordant results (explored in paper III). Therefore, we acknowledge the uncertainty of the classification of the MTB infected children, but the diagnostics we used are the best tools available (as well as T-SPOT) and generally accepted to diagnose MTB infection.[54, 248]

There are some additional evident pit-falls when it comes to classification of MTB infected children without overt TB disease. First, as the sensitivity of TST and QFT for active TB was low, there is a fair risk that some TST/QFT negative children were truly infected and wrongly classified as uninfected controls. Second, it is a risk that some of these children may have true TB disease without the typical CXR changes. A low threshold for referral could have resulted in children undergoing investigations before evident disease manifestations had occurred, or the TB foci could be extrapulmonary. Interestingly, 2 out of 4 of the smear/culture positive TB cases came

from the passive surveillance group compared to 1 out of 9 smear/culture negative (probable) TB cases. This may indicate that culture negative smear/culture negative TB cases passed undiagnosed in children with passive surveillance. Alternatively, there was an over-diagnosis of clinical TB in children with active surveillance. We think the first scenario to be the most likely as under-reporting of TB cases in children is very common.[249]

We are aware that misclassification for any reason is likely to have reduced the statistical power of our analyses and hereby reduced the true differences in biomarkers between the groups. Nevertheless, major misclassification would tend to produce a null result when comparing groups.

5.1.3. Random and systematic errors in data collection (paper I-IV)

Random and systematic errors can cause false estimates and/or confounding.
The methods used for data collection in paper I-IV, possible errors that can arise, and measures taken to minimize them, are listed in the Table 11:

Table 11

	Possible errors		Means of assessment
	Random error	**Systematic error**	
Common for all			Data collected during NCS/ACS were doubled entered applying Standard Operating Procedures (SOPs). All staff members were certified after going through a Professional Development Programme including clinical research and management, consent, basic epidemiology and infectious diseases. Staff members were also trained and certified according to standard SOPs for relevant procedures
	Errors in typing/registration	Systematically less/more errors by some staff	
Data from health/study documents			See *Common for all*
Data from interview			See *Common for all.* In addition use of the local language and structured questions. By interview, the ability to verify whether the questions are correctly understood was maintained. Using staff from the local community (outreach workers and field supervisors) to obtain this information and the informed consent.
	Misunderstandings by the respondent and/or observer	Misunderstandings dependent on the educational level/communication skills of the respondent and/or observer	
		Under-reporting of low socioeconomic class (education, income, housing, fuel)	
	Inaccurate report of duration/character of symptoms/TB exposure	inaccuracy in reports of symptoms/TB exposure is likely to increase with time	Nurses/doctors were used for interviews regarding clinical history and symptoms.

Data from anthropometric measurements			See *Common for all*.
	Errors in devices for measuring weight	Error in calibration of devices used for measuring weight.	
		Weight rounded off upwards/downwards/to the nearest 100g	Training and certification in relevant SOPs
	Errors in the application of the method for measurement	Observer-related differences in the application of methods (e.g. weight with/without diapers, time of day, relation to meal, etc)	Training and certification in relevant SOPs
Performance of the TST		Observer-related differences in the application and reading technique of TST	See *Common for all*. Training and certification in relevant SOPs
Laboratory methods (described in more detail in the text)			
	Inaccuracy in measurements	Drift in the automatic readers used (ELISA, sequencer, LSR II)	Regular standardization (of the mean/peak area/standard curves). Calculation of CV%* gives an indication of the accuracy
Microbiological samples			
	Random contamination	Non-random contamination due to different batches (ex: hypertonic saline/ medium) or staff on duty.	Training and certification in relevant SOPs. Procedures of quality assurance in the sampling and handling of lab specimen
QFT and 10-plex assay		Non-random differences in the sampling and handling of specimen	Training and certification in relevant SOPs (QFT, n=x, 10-plex, n=1)
dcRT-MLPA			
	Technical errors throughout the protocol	Non-random differences between batches/personnel	All samples were handled by the same person and supervised by another person (less strict for RNA isolation)
Flowcytometry			
	Technical errors throughout the protocol	Batch- or personnel related differences	All samples were handled by the same person
	Errors in the readings of LSR II (plugging, software)		Continuous supervision of flow runs
	Errors in gating and/or handling of data	Drift in gating cut-off with time	Gating on all batches were synchronized

*CV% or the coefficient of variance, is defined as the variance in percent for repeated measurements of the reference sample compared to the standard curve

5.1.3.1. Microbiological samples (paper I and II)

A relatively high number of children had positive NTM cultures. The TB Trials Study Group was greatly concerned that this was due to contamination, for example of the water used for gastric lavage. Therefore, an extensive review of the samples and sampling procedures were conducted in cooperation with a visiting US-based quality assurance team. These investigations, together with the non-random distribution of positive NTM cultures supported our conclusion that NTM presence was not a result of contamination: Firstly, NTM were isolated from both induced sputum and gastric

lavage samples. Secondly, NTMs were less likely to be isolated at younger ages (adjusted OR 0.21, CI 0.09-0.49 for 0-5 months, OR 0.49, CI 0.30-0.79 for 6-11 months, and OR 0.71, CI 0.47-1.01 for 12-17 months), which likely reflects an increasing interaction with the environment with age, since NTMs are ubiquitously found in soil and water. Our data provided no evidence that the presence of NTMs were related to disease (as defined by ATS/IDSA[250] in the referred children.

5.1.3.2.QuantiFERON Gold In-Tube (paper I-IV)

The processing of tubes and ELISA plates was performed manually by a limited number of trained technicians (3). No kit independent control was analyzed to assess the variability/reproducibility between plates or personnel. The coefficient of variance (CV%, defined as the variance for repeated measurements of the reference sample compared to the standard curve in percent) is likely to be greater in peripheral wells.[251] While this phenomenon may affect the interpretation of individual samples, it contributes to random error in a study-context with a high number of samples analyzed in randomized order. Accordingly, such variation is unlikely to have caused systematic bias affecting study results.

Studies on the QFT assay which elucidate the contribution to the total sample variability caused by 1) the manufacturer (between-lot variation), 2) preanalytical sources (time of day for blood draw; blood volume; intensity of shaking the tubes; delay in incubation; incubation time), 3) analytical sources (within/between-run, between operator and between laboratory imprecision), and 4) immunological sources (boosting by PPD; modulation by PAMP) have recently been reviewed by Pai et al.[65] The authors conclude that the manufacturer and the users of the test can minimize the systematic sources of variability by standardization, whereas random sources of variability are unavoidable and must be accounted for when interpreting the results. Notably, the total variability in the test needs to be determined in order to establish an appropriate test cut-off and borderline zones.[65] Regarding boosting of the QFT response by intradermal injection of PPD as part of the TST, evidence suggests a potential for boosting if blood is drawn from 7 days to 3 months after PPD administration. Boosting does not seem to occur within 3 days post-TST (equals the time point for TST reading) or later than 3 months, but interestingly seems more prevalent among subjects who are already QFT positive.[252, 253] To avoid the effect of

boosting, the NCS/ACS study protocols assured that blood for the QFT assay was drawn on the same day as the administration of the TST.

Paper I-III

In the NCS TST was performed per protocol at study day 360 (active surveillance, only) and at study closure (day 720). In addition TST was part of the diagnostic procedure at the CVW. If TST and QFT were performed within 3 months either as part of the pre-scheduled follow-up or at a previous visit at the CVW, the tests were not repeated. Visits including TST and QFT results were preferred for inclusion in the data sets if the clinical diagnosis remained unchanged from the previous visit (see section 3.3.1, paper I). We therefore conclude that the risk of false positive QFT results as a result of TST boosting was minimal in paper I-III.

Paper IV

In this paper the study participants selected from the ACS were classified based on repeated QFT measurements. All ACS participants had a TST performed at inclusion, mid-study (day 360, termed *QFT baseline* in paper IV) and at study closure (day 720, termed *after 1 year* in paper IV). None of the study participants included in paper IV had TST performed outside the pre-scheduled follow-up, thus the time interval between TST and the subsequent QFT was 12 months. Boosting of QFT by TST therefore seems unlikely.

Another question relevant for paper IV is whether subjects classified as reverters had true QFT reversion or just minor fluctuations around the test cut-off due to variability in the QFT assay itself (personnel and equipment) or biological within-subject variability as discussed above. In a review addressing within-subject variability in the QFT-response, only 3 studies fulfilled the inclusion criteria.[253] Two of these studies were conducted in high endemic settings where absence of MTB re-exposure was assumed because of short test intervals (1-3 weeks). Highest variation in IFNγ-release (± 80%) was found by van Zyl-Smit et al who measured QFT in the same subjects 3 times at 1-week intervals.[252] Among consistent positives, 19 of 21 had QFT values ≥0.70 IU/ml, a suggested upper limit for a "grey-zone" (0.35 to 0.70 IU/mL) in test interpretation,[254] whereas 18 of 21 reverters had either a baseline value ≥0.70 IU/ml

or a reduction in IFNγ-release of ≥80% from baseline to the reversion one year later, suggestion true test reversion.[252]

5.1.3.3.dcRT-MLPA (paper II and III)

All samples were run in duplicates and averaged, to reduce the risk of outliers affecting the result. The standard deviation of the reference gene (GAPDH) in all study subjects was 52%, thus slightly above the normal range for the standard deviation (35-45%) (see section 3.8.3). The least standard deviation was found for B2M (36%) but we chose not to use this as the high signal intensity in sequencing the HEX (green) labeled PCR products caused some problems in the definition of the peak. Anyway, we did conduct all analyses using B2M as the reference gene without any consequences for the results. To further evaluate the assay in our hands, the CV% of every gene from the PCR 6 runs were calculated based on peak area data of the positive control (synthetic oligonucleotide) after normalization to GAPDH CV% = St.dev/Average*100) (Table 12).

FAM labeled		**HEX labeled**	
Gene	CV%	**Gene**	CV%
CD8+A	10,6	IL10	**28,8**
CXCL10	14,2	BPI	13,0
CD4+	**30,6**	CCR7	**22,5**
IL4d2	**16,3**	SPP1	**24,8**
BLR1	12,5	CCL22	**30,1**
SEC14L1	**17,0**	MMP9	**20,3**
GUSB	8,9	RAB24	**24,1**
TIMP2	8,5	CD163	**23,5**
CCL19	5,4	TGFB1	**23,5**
FPR1	12,7	TNFRSF1B	**27,4**
IL4	13,6	FCGR1A	**24,6**
NCAM1	9,3	TNFRSF1A	**21,5**
FOXP3	**24,4**	RAB13	**29,1**
CTLA4	**30,8**	CD3E	14,8
TNF	11,4	CD19	**16,9**
RAB33A	7,1		
ABR	13,3		
TGFBR2	7,3		
IL7R	14,1		
BCL2	8,8		
CASP8	3,7		
TNFRSF18	**33,7**		

Table 12. CV% of the genes included in the dcRT-MLPA assay. %CV >15 is bolded.

Notably, for diagnostic tools a CV%>15% might not be acceptable, but with an exploratory purpose as in our studies, a higher CV% can be tolerated as long as the uncertainty in the results are taken into account in the interpretation of the results.

5.1.3.4.10-plex ELISA assay

No reference sample was included in the 10-plex ELISA assay. Therefore, the CV% based on independent controls cannot be measured. As a proxy for variation of the measurements on the six plates, the mean fluorescent intensity (FI) from duplicates of every of the eight dilutions of the kit standard which included each of the 10 cytokines/chemokines, was analyzed (Table 13).

Table 13. CV% of the standard dilutions (S1-S8) across 6 plates. Duplicates of each standard run on 6 plates were averaged before the averaged values from the 6 plates were used to calculate mean and standard deviation. For cytokines reported to be significantly or borderline differentially expressed between groups in paper II and/or paper III, the CV% value for standards with concentrations most closely matching the median concentration of the cytokines in the study samples are **boldfaced** to facilitate interpretation.

Standard	Hu IL-2	Hu IL-5	Hu IL-6	Hu IL-8	Hu IL-10	Hu IL-13	Hu IFNγ	Hu IP-10	Hu MCP-1	Hu TNFα
S1	3,9	6,9	**17,8**	**7,6**	9,9	8,8	17,5	34,1	**20,8**	12,0
S2	6,0	99,6	**16,7**	13,1	9,6	5,0	5,5	**23,7**	**10,9**	15,4
S3	4,5	12,6	16,5	14,2	10,4	5,1	**5,0**	**20,4**	10,0	**12,4**
S4	20,5	27,4	69,7	49,1	37,3	14,3	**52,6**	78,2	30,5	**48,2**
S5	6,2	10,7	16,6	14,3	**9,9**	5,5	27,3	21,3	8,9	13,9
S6	**4,0**	7,6	15,3	10,2	**10,6**	**1,7**	57,4	20,3	8,4	11,6
S7	**10,2**	**11,0**	19,4	15,5	9,2	**5,2**	48,3	21,0	9,0	13,4
S8	21,6	8,2	15,5	31,0	9,9	2,8	24,5	59,9	10,0	12,6

As illustrated in Table 13, CV% of the standards with a concentration most closely corresponding to the study samples was ≤15% for IL-2, IL-5, IL-8, IL-10 and IL-13 whereas the CV% for IFNγ and TNFα was as high as 50%. CV% calculations based on more than 6 samples could have given a more satisfying CV%. We feel that the exploratory nature of paper II and III permits less stringent criteria with regard to assay validation, but high variability is likely to have reduced the power of statistical analyses, and must be taken into account in the interpretation of the results. In future studies, CV% should certainly be evaluated through kit-independent controls.

5.1.3.5.Flow cytometry (paper IV)

As flow cytometric data were obtained in batches within a period of 8 weeks, a drift in the LSR II flowcytometer could cause systematic errors. During the study period the laser alignment, laser area scaling and parameter voltages on the LSR II flowcytometer were calibrated according to standard beads as part of the standard

routine, by the chief manager Kirsti Landsverk at the core facility at Oslo University Hospital, Montebello. The CV% for the standard beads are given in Table 14.

Month	CV% for standard beads LSR II, 2012			
	FITC	APC	PE	Pac Blue
January	2,1	3,8	1,9	2,8
May	2,2	2,4	1,8	1,6
June	1,8	2,8	2,0	1,7
August	1,8	3,0	2,1	2,0
September	1,8	2,6	2,5	2,6
October	1,3	2,8	1,7	2,1

Table 14. CV% for standard beads run on all 4 lasers on the LSR II, Montebello in 2012. The period for analyses of the samples for paper IV is marked in grey.

In addition, compensation controls (separate beads for all included fluorochromes) where run before every experiment. While voltages for all channels were left unchanged after calibration, acceptable variability was registered for fluorochromes with ≥15% spectral overlap (Table 15).

Table 15. Spectral overlap >15% after automatic calculation of compensation

Fluorochrome	minus % fluorochrome	30.4.12	15.5.12	22.5.12	29.5.12	7.6.12	11.6.12	13.6.12	Variability (%)
Alexa 700	PerCP-Cy5.5	27	27	22	32	32	32	33	16,6
PE-Cy 5	PerCP-Cy5.5	27	27	27	29	27	27	27	0,6
Alexa 700	APC	31	31	30	29	31	31	32	0,9
PE-Cy 5	APC	31	33	36	28	26	26	27	15,0
APC Alexa750	Alexa 700	27	27	27	27	28	28	28	0,3
PerCP-Cy5.5	PE-Cy 5	39	40	40	39	40	41	40	0,5
APC	PE-Cy 5	27	26	22	33	33	32	32	18,6
APC Alexa750	PE-Cy7	18	17	14	21	22	22	23	11,0

5.1.4. Selection bias and confounding

Selection bias is a systematic error which occurs when subjects are systematically left out of the study or drop-out, because of the procedures used for study inclusion or participation.[255] With regard to establishing the incidence of TB, which was the primary aim of the NCS and ACS, selection bias could have been introduced if for instance; literacy was required to get adequate information about the study and/or complete questionnaires; information was given and/or interview undertaken in another language than the local or; only neonates having a supervised birth were included. All these factors disfavors enrollment of subjects from low socioeconomic classes. As the TB incidence is highest in underprivileged populations this selection bias can cause wrong estimates of TB incidence. Another possible selection bias

would be if a sub-group sharing genetic background with higher or lower susceptibility for TB than the remaining population, systematically rejected enrollment due to a cultural reluctance to give blood or undergo certain examinations.

Confounding can be explained as "a confusion of effects". This implies that the measured effect or association is not related to the analyzed variable but totally or in part, related to other unknown factor(s) differentially distributed between the groups compared.[255]

Considerations on possible selection biases and confounding are addressed separately for paper I-III and paper IV.

Paper I-III

Of eligible neonates, 90% were enrolled in the NCS (section 3.2.1.1). This corresponds to the upper goal for enrolment set in the study protocol (80-90%). Of enrolled subjects ~90% participated throughout the study. The frequency of participants who did not complete the study was similar within the active and passive surveillance groups (no significant differences between the groups), as well as for the whole NCS cohort, are listed in Table 16.

Table 16. Study adherence	Surveillance		NCS cohort
	Active	Passive	
Participants not completing the study due to:			
Death	49 (2.2)	71 (3.3)	120 (2.7%)
Lost to follow-up	38 (1.7)	60 (2.8)	98 (2.2%)
With-drawn	93 (4.2)	111 (5.1)	204 (4.7%)
Fulfilled the study	2034 (91.9)	1925(88.8)	3959 (90.4)

The 746 children referred to the CVW during the 2-year surveillance constitute the source population of paper I-III. 10 (1.3%) of these children withdrew from the study after the first visit to the CVW and additionally 9 (1.2%) children were lost to follow-up. These numbers are small, but could have a confounding impact on our results if these children were predominately TB cases. We find it unlikely that symptomatic children should predominately be lost to follow-up as these are most likely to profit by the health care provided by the study.

Some data values are missing as sampling was omitted as a consequence of clinical evaluation (e.g.: blood-draw in very anaemic or seriously ill children), whereas other variables might be missing due to technical errors or because the procedure/question were missed by the staff. Nevertheless, the number of missing values within each variable were marginal (<2%) except for haemoglobin values (104, 13.9%). Systematic analyses to clarify systematic differences in socio-demographic or clinical variables between drop-outs and the remaining study population are currently being conducted as part of a manuscript in preparation by the TB Trials Study Group on TB incidence in the study area.

It is evident that most of the children referred to the CVW were from the active surveillance group, demonstrating a reduced threshold for seeking health care in children under active surveillance. While there were no difference in the proportion of children under active and passive surveillance for children with and without clinical TB (Table 1, paper I and II), the proportion of probable TB was higher in children under active surveillance consistent with a lower threshold for referral (Table 2, paper I). This is further discussed in section 5.2.

TST ≥10mm outcome was both a referral criteria and 1 of 4 diagnostic criteria (≥1 criteria in addition to characteristic CXR changes) required for a diagnosis of clinical TB. The TST estimates could therefore be subject to selection bias, which is a limitation in paper I. This compromise in design was required in order to avoid losing TB cases; the primary aim of the NCS was to establish the true incidence of TB. This selection bias could result in an overestimation of the TST's sensitivity for clinical TB, but as none of the probable TB cases depended on a positive TST for their diagnosis, this does not affect the TST sensitivity estimate. Furthermore, compared to the OR estimates of the associations between a positive TST and the assessed variables, the OR estimates for a positive QFT outcome may be overestimated because TST-QFT+ discordant positives children within the NCS were only referred if they had known TB exposure or were symptomatic, whereas TST+QFT- children might have had no other referral criteria. Notably, the effect is probably limited as referral based on a positive TST only was present in 51 (6.8%) of 746 children of whom 44 (86.3%) were referred at study closure.

In paper II and III, uninfected controls were selected in order to make the clinical groups in paper II as equal as possible with regard to sex and age. For each child with clinical TB and for each child with MTB infection, a sex matched subject as similar in age as possible, was selected. Opposing this age- and gender matched selection, differences between the 3 clinical groups were analysed by pairwise comparison, not with matched statistical methods. This approach could theoretically have introduced a systematic selection bias. From a statistical point of view it might have been better to pick the uninfected controls randomly since analyses adjusted for sex and age were applied anyway.

Paper IV

Possible selection biases in the ACS is not included herein, as paper IV represents an exploratory laboratory study in which this has little direct relevance. Study subjects for paper IV were picked by computerized randomization among subjects enrolled in ACS with available PBMCs. We argue that this was the best method to select study participants to avoid selection bias. Nevertheless, there might be systematic reasons for PBMCs not being available. Similarly, as we experienced problems with bacterial contamination in about 20% of the vials, this might have resulted from a systematic error in the collection of specimen, which could theoretically result in a selection bias.

5.1.5. Statistics

5.1.5.1. Presentation and definition of independent variables (paper I)

In paper I, Table 1, the distributions/proportions of independent variables are presented for all children referred and classified according to the TB diagnostic algorithm. Some variables are presented both as continuous and categorical variables to get a better picture of the data: birthweight; age (months); WHO Child Growth Standards Z-scores;[209] hemoglobin; TST; QFT. In evaluating socioeconomic variables, factors which indicates low socioeconomic status (SES) are normally regarded as exposure, thus higher SES are usually used as reference. In our data, low numbers in the high SES categories made it more statistically correct to use low SES as reference. The categorizations and definitions of socioeconomic variables are described in section 3.9., paper I). WHO Child Growth Standards Z-scores are presented as mean/min-max as well as the proportion of children with Z-score <-2.

This is the standard cut-off for moderate to severe malnutrition. The adequacy of new cut-offs for the dichotomous nutritional variables introduced in univariate and multivariate logistic regression analyses (Table 3 and 4, paper I) could be a matter of dispute. We argue for this choice in paper I, and more in-depth in section 3.9., paper I). In addition to the argument given there, we want to underline that it is unknown whether the WHO cut-offs of <-2 or <-3 are adequate in evaluating immunological changes in the malnourished individuals. Furthermore, we concluded not to use continuous nutritional variables as the relationship between malnutrition and immunological outcomes is more likely to be U-shaped than linear. This is because over-weight is associated with increased inflammation which implies altered immune responses.[256] This effect is presumably marginal in our study as only 8 children had a BMI-for-age score >2. Nevertheless, as false interactions can be a problem with continuous variables, our argument for a dichotomized variable was further strengthened.

Regarding malnutrition in children enrolled in the NCS but not referred to the CVW and therefore assumed to have lower morbidity, referred children were slightly more malnourished. In general, Indian children have lower gestational age-specific birthweight[257] and reduced growth (height and weight)[258] compared to WHO Child Growth Standards.[209 209, 217] A recent paper analyzed the distribution of the Z-scores obtained from the National Family and Health Survey (NFHS) which included information on >27,000 children <3 years in rural India in 2005.[259] For this population the mean height-for-age Z-scores (HAZ) was -1.59, whereas the mean weight-for-height Z-score (WHZ) was -1.08. The Z-scores tended to be lower with increasing age from birth, until stabilization at about 18 months. Children included in paper I, had at a mean age of 14.8 months, mean Z-scores of -1.25 (HAZ), -1.80 (WHZ) and -1.95 (WAZ). For NCS children not referred to the CVW, we have weight data from the children under active surveillance at 14 and 16 months of age. This enables estimation of WAZ score for comparison with referred children. Assuming a linear relationship between WAZ and age, non-referred children of 14.8 months, would have an estimated WAZ about -1.32 (Figure 16). Subsequently, referred children had a WAZ score about 30% lower than non-referred children. This confirms the assumption of referred children having a poorer health condition than the un-referred despite a low threshold for referral.

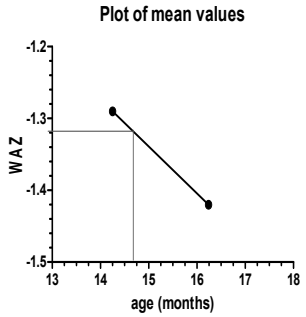


Figure 16. Estimate of WAZ score in children enrolled in the NCS who were not referred to the CVW, at 14.8 months of age. (A linear relationship between WAZ and age is assumed)

5.1.5.2. Selection of variables for adjusted analyses (paper II and III)

The aim of paper II and III was exploratory. Independent variables listed in Table I (paper II and III) were evaluated by their confounding potential and subsequent inclusion in adjusted analyses as described in section 3.9 (subtitle; Paper II). Age, WHZ, WAZ and NTMs in culture were adjusted for. In the light of the findings in paper I, two aspects could have been assessed differently: First, the WHO cut-off (<-2) for the Z-scores, might not be optimal when adjusting for malnutrition in this population. Second, including both WHZ and WAZ in adjusted logistic regression could introduce problems related to collinearity as indicated by analyses performed as part of paper I (see section 3.9., paper II and III) (Spearman's rho=0.51, p>0.0005, Tolerance=1.0, VIF=1.0). The risk of collinearity might have been less prominent if WHZ and HAZ were used for correction (Spearman's rho =0.21, p=0.0003, Tolerance=1.0, VIF=1.0). Alternatively, WHZ could be used alone, as this variable was most unevenly distributed.

5.1.5.3.Considerations on multiple testing (paper II and III)

In paper II and III, no correction for multiple testing was done in single gene analyses due to the exploratory nature of the papers. Methods correcting for multiple testing (Bonferroni correction and false discovery rate (FDR) control) was applied in the initial analyses, but revealed minimal differences between the groups. We consider this likely due to large heterogeneity within the groups (see section 5.1.2). Instead, to avoid conclusions based on false differences (Type I error); in paper II, we restrict our conclusions on differences to immune biomarkers that turn out significant in more than one pair-wise comparison. This is true for the transcription of RAB33A which is lower in children with clinical TB compared to either MTB infected children or

controls, and for the transcription of TGFB1 which is higher in children with either clinical disease or MTB infection compared to controls. Multiple testing is not required in the global test[223] nor in lasso regression[224]. In paper III, we cannot exclude type I errors with regard to single biomarkers, but as discussed in the paper, the tendencies of the immune biomarker profiles in terms of pro- or anti-inflammatory or blunted patterns seem relatively consistent between different comparisons reported in the study, and the findings makes sense in the context of current literature.

5.2. Discussion of main results

Sited from section 2: *"The general aim of this thesis was to contribute to the understanding of current immunological diagnostic and explore the diagnostic potential of new immune biomarkers in Mycobacterium tuberculosis infection and disease"*. This was undertaken in the setting of the two described cohorts studies; NCS (paper I-III) and ACS (paper IV). These cohorts represent two important target populations for future TB vaccines: neonates as targets for a better pre-exposure vaccine ("Improved" BCG), and adolescents as targets for post-exposure vaccines when the effect of BCG is waning.[260] There are two important distinctions between the study subjects selected from the NCS and the study subjects selected from the ACS: Firstly, the most obvious difference is in age: NCS children selected for paper I-III are <3 years, whereas ACS children selected for paper IV are 12-18 years. Secondly, the children in paper I-III are symptomatic whereas the adolescents in paper IV are healthy.

Another distinction between the papers worth noting: while a clinical TB classification of the study participants forms the basis for the analysis approach in paper I and II, paper III and IV explore immune biomarkers in study participants classified uniquely by their TST and/or QFT results: In paper III, the children are classified by their TST and/or QFT. In paper IV, healthy adolescents were classified based on 2 QFT results 1 year apart.

Regarding the clinical condition of the study subjects, all the children in paper I-III were evaluated for suspected TB due to known TB exposure, symptoms and/or TST ≥10mm (see section 3.3.1.). Of the 746 children admitted to the CVW, 655 (88%)

were symptomatic with failure to thrive (FTT), persistent fever and/or cough. Anthropometric measurements support that these children were in a poorer health condition than children within the active surveillance arm of the NCS in whom TB was not suspected during the follow-up (mean WAZ -1.95 versus -1.32 at the age of 14.8 months, see section 5.1.5.1.). With regard to MTB infection; children this young are most likely within the course of primary infection as it takes 1-3 years for TB lesions to calcify. Calcified lesions are considered the hallmark of a latent stage of MTB infection.[261] (For paper I, all the 746 children were eligible whereas 210 of the 746 were selected for the biomarker sub-studies in paper II and III (see section 3.3.1).) On the other hand, paper IV includes 52 healthy adolescents in whom data were collected at pre-scheduled follow-up visits within the active surveillance arm of ACS (see section 3.3.2.). These subjects had no symptoms and only one had known TB exposure. Therefore, in summary, immune responses in the adolescents can be assumed to differ importantly from immune responses in the young children in three aspects: 1) the immune system has reached a more mature state,[81, 262] 2) the immune system is not affected by ongoing infectious intercurrent diseases [221] and 3) if MTB infected, they are much more likely to have entered a latent stage.[22] These considerations are important to keep in mind throughout the following discussion.

The results in paper IV are discussed first; this is because healthy MTB infected adolescents in many ways represent a referral group when it comes to immune responses to MTB; their immunity is neither affected by likely intercurrent disease nor immaturity due to young age. Notably, the MTB infected adolescents were categorized in two groups assumed to have different risk of TB progression judged by their longitudinal QFT results: QFT consistent positives and QFT reverters. QFT reversion can be assumed to represent reduced risk of TB progression compared to consistent positives.[263] This is supported by the following evidence: 1) IGRA-reversion is more likely in subjects with a negative TST[30, 227, 254, 264, 265] and/or IFNγ-responses close to the test cut-off.[254, 265] 2) The risk of TB progression seems to increase with the magnitude of MTB-specific IFNγ-responses (reviewed in[65]). 3) Adults with concordant positive TST and IGRA result have increased TB risk compared to subjects positive for either test (discordant test results).[232] As expected, reverters in our study had significantly reduced QFT and TST responses compared to consistent positives. Whereas more than half of the consistent positives showed a

positive TST at both time points, all reverters had a negative TST at baseline and one reverter only, had a TST ≥10 mm after 1 year. Therefore, the assumption of reduced risk in QFT reverters in this study seems appropriate. (An argument for true test reversion in the study subjects have previously been argued for in section 5.1.3.2., paper IV.)

Based on the assumption that the quality (polyfunctionality) of the T cell response is of greater importance for TB protection than the total amount of specific IFNγ-production,[117, 118] polyfunctional T cells have been suggested to be protective.[117, 120, 266, 267] We therefore hypothesized that QFT reverters differ from QFT consistent positives with regard to the quality and magnitude of MTB-specific T cell responses in peripheral blood. Relative frequencies of PPD-specific polyfunctional (IFNγ+IL2+TNFα+) CD4+ T cells were high but similar in both QFT consistent positives and reverters at baseline. Reverters displayed lower absolute frequencies of these cells, which were further reduced 1 year later. Notably, the polyfunctional subset was most efficient in terms of cytokine production and their absolute frequency correlated well with the magnitude of the QFT-response. Subsequently, our data do not support that the relative or absolute frequencies of PPD-specific polyfunctional CD4+ T cells in peripheral blood can explain the reduced risk of TB progression observed in QFT reverters. PPD is a relatively crude mixture of mycobacterial antigens shared by the BCG strain and NTM. Nevertheless, we observed a good correlation between absolute frequencies of PPD-specific T cells (when assessing either all IFNγ-producing subsets or the polyfunctional subset) and the magnitude of QFT-response. As the IFNγ-release in the QFT assay depends on T-cell recognition of RD1-specific antigens (mostly; TB7.7 is not a RD1-antigen) not present in the BCG-strain and most NTM, the polyfunctional T-cell responses to PPD measured here likely reflects specific anti-MTB immune reactivity. Further supporting this: The TST which also applies the PPD, is not affected when the BCG vaccine is administered at birth and not repeated.[65] The individual BCG status of the study subjects in paper IV are given in Table 17. Comparing QFT consistent positives with reverters, significantly fewer were vaccinated (16/20 versus 21/21, Fisher's Exact test, p=0.048), whereas there was no difference in the absence of a BCG scar.

Table 17. BCG status in paper IV study subjects	QFT group			Total n=51(%)
	Consistent positives n=21(%)	Reverters n=21(%)	Consistent negatives n=10(%)	
BCG vaccinated	16* (80%)	21 (100%)	9 (90%)	46 (90.2%)
Presence of BCG scar	12 (57.1%)	17 (81%)	7 (70%)	36 (69.2%)

*n= 20; one subject missing due to unknown BCG vaccination status

Moreover, both QFT reverters and controls had negative TST at baseline, and controls from the same study-population/vaccine coverage, had negligible absolute frequencies of PPD-specific CD4+ T cells. This support little impact of BCG-vaccination on mycobacterial-specific T cell responses. In the light of TB risk, we find the close correlation between absolute frequencies of PPD-specific polyfunctional T cells and the magnitude of the QFT response particularly interesting: In non-human primates the magnitude of the IGRA-responses reflects the antigen load.[188] There is broad consensus that the higher load of MTB bacilli within the organism, the higher the risk of TB progression.[1, 188] Interestingly, a recent study found no correlation between mycobacterial growth inhibition and the magnitude of IFNγ ELISPOT responses.[268] Notably, no correlation between the frequencies of polyfunctional T cells and protection against TB was found in BCG-vaccinated infants.[121] Subsequently, rather than being markers of protection, we suggest that absolute frequencies of PPD-specific polyfunctional T-cells reflects antigen load. Our data also supports earlier speculations on the requirement for persistent or recurrent MTB exposure to maintain specific IFNγ-responses.[30, 53, 227, 264, 269]

Even if true in healthy MTB infected subjects, evidence suggests that the relationship between antigen load and the magnitude of the IFNγ-response is altered in subjects with severe TB disease.[270] This could be explained by sequestration of immune cells in the tissue,[271, 272] as well as relative immune-deficiency in severely ill individuals.[273] Very few of the young children with clinical TB in our material, had positive immunological tests (4 out of 13, TST sensitivity 31%, QFT sensitivity 23%). Both tests were negative in the only child with a positive smear (grade 3+ in gastric aspirate, MTB confirmed by PCR). Of the 9 unconfirmed TB cases, only one had a positive TST (16mm). This child also had symptoms, and was therefore not subject to

selection bias by the diagnostic algorithm (see section 5.1.4). Knowing this, we hesitate to suggest that TST-/QFT+ discordant children in paper III have reduced risk of TB progression compared to concordant positive children as evidence suggests for adults.[232] Furthermore, in the context of paper IV: While reduced antigen load seems to be a reasonable explanation for reduced absolute frequencies of polyfunctional mycobacterial-specific CD4+ T cells in adolescents who reverted to QFT negative (all were TST-/QFT+ discordant positive at baseline), a TST-/QFT+ discordant result in symptomatic children <3 years could be a marker of blunted immune responses from young age and/or malnutrition. In that case, these children could have an increased risk for TB progression. The findings in paper I support this, but we acknowledge that an alternative explanation could be that a TST-/QFT+ discordant test result from a more recent MTB infection,[268] as the TST response take weeks to develop.[68]

In paper I, we address a fundamental challenge in diagnosis and adequate management of young children; the high proportion of bacteriologically unconfirmed TB cases. While TST and QFT are used as supplements in the diagnosis of pediatric TB, we report, in consistence with others, a low sensitivity for these tests in clinical TB.[63, 201, 274] It is rather striking that 8 out of 9 bacteriologically unconfirmed TB cases were negative for both tests, but reduced sensitivity of TST and IGRAs in this group compared to smear/culture positive TB cases has been observed by others.[245] A major question is therefore whether bacteriologically unconfirmed TB cases which constitute 2/3 of clinical TB cases in most pediatric studies,[75] are true TB cases. Epidemiological estimates strongly suggest that they are.[12] Assessing risk-factors and TST/QFT responses in bacteriologically unconfirmed TB cases compared to smear/culture positive TB cases would have been the most straight forward approach to shed light on clinical and immunological features characteristic for each of these groups of patients. Unfortunately, there were a limited number of TB cases in our study. Therefore, we explored instead clinical, demographic and nutritional (intrauterine and post-natal) factors that might be associated with TST and/or QFT outcome. Factors associated with TST and QFT outcomes might indirectly contribute to the understanding of host immune responses. We reported that children <2 years were less likely to have a positive TST and/or QFT and more likely to have an indeterminate QFT. If the sample volume is adequate, indeterminate QFT results are most often caused by low mitogen responses (QFT Mitogen minus Nil < 0.5

IU/mL),[275] which was also the case here, although not reported in paper I: bivariate analysis of age and mitogen response as continuous variables, revealed a moderate positive correlation between age (months) and the magnitude of the mitogen response (Spearmans Rho= 0.34, p<0.0005), an association supported by others,[276, 277] but the analysis is restricted by the lack of values >10IU/mL, a penalty of the QFT assay. Previous studies in young children are contradictory on whether there are positive associations between age and TST[243, 278, 279] or QFT result,[274, 278-280] but the largest study support such associations.[278] These associations likely reflect a cumulative exposure with age, but might also reflect that immunological tests perform differently in subjects with an immature immune system. The latter is supported by reports on determinants for an indeterminate QFT, where exposure is irrelevant. Many studies support that indeterminate results are more frequent at young age.[231, 274, 275, 277, 278, 280-282] In our study, children with stunting due to chronic malnutrition (height-for-age Z-score within the lowest quartile), seemed more susceptible to MTB infection and more prone to indeterminate QFT. Malnourished children are in general more susceptible to infections [283] because of altered immune responses,[85] but to our knowledge, no previous studies have established this for MTB infection by the use of height-for-age Z-score. TST proved less reliable in children with wasting (weight-for-height Z-score within the lowest quartile). The literature on the impact of malnutrition on the TST response is contradicting,[284-287] but we suggest that this is due to considerable variation in how malnutrition is defined. In addition, if the Z-scores are used, the WHO cut-offs of <-2 (moderate to severe malnutrition) or <-3 (severe malnutrition)[209] are not defined with regard to the impact of malnutrition on the host immune response. We also found that symptomatic children (fever and cough) were more likely to have indeterminate QFT responses. Similar reports exists,[221] and this findings likely reflects the general impact on the immune response by ongoing disease regardless of etiology. Our findings shed light on the poor sensitivity of TST and QFT in MTB infection and disease in a pediatric context. Ideally, determinants for the TST and QFT outcomes should be evaluated in clinical TB cases, but this is barely doable in community-based settings with low to medium TB incidence. The fact that the majority of bacteriologically unconfirmed cases in our study (8 out of 9) came from the active surveillance arm, suggests that unconfirmed cases represent either an early phase in TB disease or a manifestation with diffuse and/or little symptoms. Evidence suggest that children <3 years might have little symptoms despite severe disease,[22, 288]

and together with the fact that children with bacteriologically unconfirmed TB in our study were younger than children with smear/culture positive TB (median age 14 months versus 22 months, not statistically tested due to low numbers), different disease manifestation in younger children related to immature immune responses are likely. Unfortunately, we cannot confirm or reject whether bacteriologically unconfirmed cases represent an early disease stage as all children diagnosed with clinical TB were referred to the Revised National TB Control Program for treatment. An alternative explanation is that young age seen in the bacteriologically unconfirmed cases, make MTB infection less likely as a shorter life represents a reduced accumulated risk of exposure. Following this, unconfirmed cases in our study would represent an over-diagnosis of TB. However, we find this unlikely as smear/culture positive TB cases only account for about 30% of clinical TB cases in the majority of pediatric settings.[75] Moreover, the evidence of an impact of age on the severity of disease manifestations[22, 26] and immune responses, are compelling. In addition, epidemiological estimates strongly suggest that pediatric TB is under-diagnosed.[12]

As discussed in section 5.1.2.1 and 5.1.2.2 we might have lost discriminatory power in paper II due to misclassification of TB status. This might explain why the differences in biomarkers exhibited by the dcRT-MLPA are much less pronounced in our study compared to the differences between adults with smear/culture positive TB or healthy MTB infected adults reported in by Joosten et al.,[211] but biomarkers with the potential to increase diagnostic accuracy must be robust. In analysis of single genes, we report that the expression *RAB33A* in unstimulated peripheral blood was significantly lower in children with clinical TB compared to children with MTB infection and controls. Down-regulation of *RAB33A* in TB is consistent with previous findings.[289, 290] RAB33A is a small guanosine triphosphatase (GTPase) suggested to be involved in the transport and fusion of intracellular vesicles.[291] Phagosomal arrest is a major survival strategy for MTB (See section 1.2.4), which might be mediated through a dysregulation of GTPases.[292] Our study also identified *RAB33A* in the adjusted lasso model as the only gene with discriminatory power between children with clinical TB and MTB infection (AUC 77.5%). *RAB33A* was also one of 5 genes with a substantial discriminatory power between children with clinical TB and uninfected controls (AUC 91.7%), and one of 11 genes with modest discriminatory power between children with MTB infection and uninfected controls (AUC 72.2%).

RAB33A has previously been identified in the context of biosignatures which enables the discrimination of confirmed TB, healthy latently infected and healthy uninfected adults of African or Caucasian origin.[290, 293] Even though differently expressed in single gene analysis, *RAB33A* was not included in the predictory biomarker signature identified by the lasso model on dcRT-MLPA data from African and South American adults.[211] Identification of *RAB33A* in Indian children with much less rigorous clinical categories strengthens the evidence that this biomarker can be used in the diagnosis and discrimination of MTB infection and disease. Another transcriptional marker identified by our analyses of unstimulated peripheral blood was *TGFB1* which had increased expression levels 1) in clinical TB compared to uninfected controls and 2) in MTB infection compared to uninfected controls. *TGFB1* encodes the cytokine TGF-β, which has immune-modulating properties in the priming of T-cells. TGF-β alone induces differentiation of Tregs, whereas in the presence of IL-6, the hallmark of inflammation, delineation is skewed towards Th17 cells.[95] Elevated levels of TGF-β have been reported in TB patients (peripheral blood) compared to healthy control with or without MTB infection.[294, 295] In addition, this biomarker took part in the 11-biomarker signature with modest discriminatory power between children with MTB infection and uninfected controls (AUC 72.2%). Increased expression levels of *TGFB1* in adults with smear/culture positive TB compared to healthy uninfected controls have been reported previously when mRNA from freshly isolated monocytes was analyzed [296], and compared to healthy latently infected adults.[294, 295] *TGFB1* was also one of 15 genes which discriminated smear/culture positive TB cases from uninfected controls (AUC 86%).[211] Supported by these findings, *TGFB1* is likely to have a diagnostic potential in the diagnosis of pediatric MTB infection and disease.

In paper III, we hypothesized that the biomarker data acquired as part of paper II could provide some insight into the immunological mechanisms associated with concordant and discordant TST and QFT results frequently found in young children. In clinical settings, it is unclear how discordant TST and QFT results should be interpreted in terms of TB risk and preventive treatment, although the predictive value of concordant or discordant results has been addressed in some studies.[232, 297] For instance; do discordant TST+/QFT- results represent increased specificity or reduced sensitivity of the QFT for MTB infection in a given setting (compared to TST)? False positive test results are least likely in concordant positive subjects, thus concordant

positive children in paper III can be assumed to be the most likely to have true MTB infection. This is consistent with known TB exposure being more frequent in these children in our study. Regarding discordant positive children, it is likely that a proportion of the TST+/QFT- results constitutes false positive TSTs since all the children were BCG-vaccinated at birth and <3 years at the time of testing.[298] Regarding the TST-/QFT+ results we find it more likely that these children are truly MTB infected as the specificity of QFT is reported to be high in similar populations.[63, 71] Supporting the assumption of a true positive QFT in these children is a median specific IFNγ-response well above the test cut-off (1.11 IU/mL). Notably, the sensitivity of MTB infection of both TST and QFT in our study population is unclear due to the lack of a gold standard.[299] In a clinical setting where MTB-infection is suspected in a child aged <5 years either TST, QFT or both test are used together with TB exposure to guide the decision on preventive treatment. We therefore assessed biomarker differences in children based on either or both tests compared to children assumed to be uninfected by MTB (TST-/QFT- concordant negative). The main immune biomarker differences between TST/QFT concordant positive, concordant negative and discordant positive children are illustrated in Figure 13.

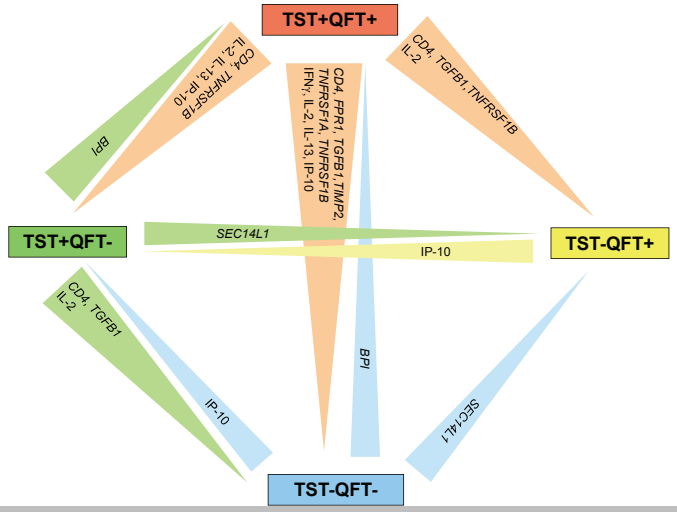


Figure 13. Immune biomarkers in peripheral blood differentially expressed between children with concordant or discordant TST and QFT test results. Biomarkers in italics represent transcriptomes analyzed directly ex vivo, whereas normal fonts represent proteins in supernatant after MTB-specific stimulation (TB-Ag tube in the QFT assay). The triangles illustrate up-regulation (broad end) versus down-regulation (pointed end) between the different groups.

had differentially expressed transcriptomes (unstimulated, *direct ex vivo*) of *FPR1, TNFRSF1A* (both up-regulated) and *BPI* (down-regulated), and increased levels of IFNγ in TB-Ag stimulated supernatants (from the QFT assay). Contrary, all test positive children had up-regulation of the transcriptomes *CD4* and *TGFB1* (unstimulated, *direct ex vivo*), and increased levels of the cytokines IL-2 and IL-13 (stimulated; from the TB-Ag tube in the QFT assay), when compared to concordant negative children. *CD4* and IL-2 are general markers of adaptive immunity: CD4+ T cells are known to be crucial in host defence against MTB (see section 1.2.3.1). IL-2 is secreted by T cells upon antigen recognition and activation through the T cell receptor, and is required for the proliferation and maintenance of antigen-specific T cell pools (memory T cells).[150] Most studies report that IL- 2 in stimulated samples is elevated in adults with TB disease compared to controls and the IL-2 levels seem to correlate with known TB exposure, but some studies report reduced levels in TB patients compared to MTB infected subjects (reviewed in[300]).The role of *TGFB1* has been discussed for paper II. IL-13, a Th2 cytokine, seems to inhibit protective IFNγ-induced autophagy in MTB infected macrophages[162] favouring macrophage necrosis[104] possibly contributing to disintegration of the granuloma (see section 1.2.4.). A previous report on IL-13 release in children with TB disease or MTB infection suggests a positive correlation between IL-13 and Th1 cytokines,[301] and increased levels have been reported in children with TB disease compared to uninfected controls.[302] Moving on to a more specific assessment; the immune biomarker profiles in concordant positive versus discordant positive children, concordant positives had higher levels of the transcripts *CD4* and *TNFRSF1A* as well as the cytokine IL-2 than both TST+/QFT- and TST-/QFT+ discordant children. Furthermore, the transcriptomes *TGFB1* and *TNFRSF1B* as well as the chemokine IP-10, are interesting: *TGFB1* because of an up-regulation in both concordant positives and TST+/QFT- discordant children compared to concordant negatives, but also an up-regulation in concordant positives compared to TST-/QFT+ discordant children; *TNFRSF1B* because of an up-regulation in concordant positives compared to concordant negatives an both the discordant groups; IP-10 because TST+/QFT- discordant children had lower levels than both concordant negative and concordant positive children, whereas concordant positive children had elevated levels compared to concordant negatives, and TST-/QFT+ discordant children had increased levels

compared to TST+/QFT- discordant children. This elevated level of IP-10 was, together with a down-regulation of the transcriptome *SEC14L1* (in TST-/QFT+) the only direct difference between the discordant groups. *TNFRSF1A* and *TNFRSF1B* encodes the membrane-bound TNFα-receptors TNFR1 and TNFR2. Binding of TNFα to these receptors mediate protective apoptosis in MTB infected macrophages,[104, 164, 303] but the receptors can also be shed by proteolysis. Shedding restricts the effect of TNFα by competing binding and reduced density of TNFRs on the cell surface (transient?).[304] Evidence suggest a shedding of these reseptors in TB pathogenesis as TNFR1 and TNFR2 transcriptomes have been reported higher in whole blood but reduced in blood monocytes of TB patients compared to healthy household contacts and controls.[165] Whether *TNFRSF1A* and *TNFRSF1B* represent increased levels of membrane-bound or soluble reseptors in our setting cannot be determined as the analyses were restricted to whole blood. IP-10, (IFNγ-inducible protein 10) belonging to the CXC-chemokine family (also named CXCL10), is secreted by monocytes and APCs upon stimulation by multiple cytokines; main IFNγ and TNFα, following the same kinetic pattern as IFNγ.[300] As the IP-10 ligand, CXCR3, seems to be uniquely expressed on T cells, IP-10 stimulation mediates a specific effect on lymphocytes,[305] creating a positive feed-back loop resulting in amplification of IP-10 levels compared to IFNγ. This probably explains why IP-10 seems to be a more potent marker of antigenic stimulus and less influenced by CD4 counts or function than IFNγ.[300] A higher amplitude of IP-10 compared to IFNγ was also demonstrated in our study as the difference in IP-10 levels between stimulated (TB Antigen tube) and unstimulated (Nil tube) samples was significant in both concordant and discordant positive children whereas the difference in IFNγ levels was only significant in concordant positive children. Somewhat surprising, IP-10 levels were higher in concordant negative children than in TST+/QFT- discordant children, but little discrimination by IP-10 when comparing active TB patients with patients with other pulmonary disease has been described,[306] and likely reflects intercurrent disease in the concordant negative children in our study. *SEC14L1* might have a role in intracellular vesicular transport and was identified by microarray analysis of differences between TB patients and healthy TB contacts,[293] and took part in the transcriptional 5-biomarker signature identified in paper II. In summary for paper III, when considering the analyses which take both TST and QFT results into account, general markers of adaptive immunity (*CD4*, IL-2) seemed to associate with a positive TST, whereas a concordant positive

result also associated with an increase in *TNFRSF1B, TGFB1, TIMP-2* and IL-13, all likely players in the pathogenesis towards TB. Whether they serve host or pathogen in this interaction remains unclear. Interestingly, when compared to concordant negatives, there was no overlap of differentially expressed biomarkers between the discordant groups, suggesting that these phenotypes represents and measures distinct immune responses. Futhermore, we were intrigued to find so little difference in IFNγ between the groups as they were categorized based, in part, on MTB-specific IFNγ-release as determined by the QFT assay. Instead, IP-10 proved to be more differentially expressed between TST+/QFT- and TST-/QFT+ discordant children. Notably, TST-/QFT+ discordant children in this study were younger and had a higher proportion of wasting (WHZ < 2SD) than the other test positive groups. It is reasonable to assume that these factors have contributed to the reduced expression of general markers of adaptive immunity (*CD4*, IL-2) seen in these children compared to concordant positives. These markers seem to associate with a positive TST since they are also elevated in TST+/QFT- discordant compared to concordant negative children. Having a positive QFT, TST-/QFT+ discordant children are very likely to be MTB infected.[71] Inadequate adaptive immunity is likely to represent an increased risk of TB progression and implies reduced sensitivity of T cell based diagnostics in these children. IP-10 seems less influenced by CD4 counts[300] and for young children, our study suggests a potential of increased sensitivity of IP-10 compared to IFNγ in a diagnostic setting and possibly in predicting TB disease.

6. CONCLUSIONS

6.1. What the studies add

6.1.1. Impact on the interpretation of current diagnostic tools in MTB infection and disease.

This thesis highlights the complexity in the interpretation of diagnostic tests in young children with suspected TB. This work provides strong arguments to the management of children: It is vital to keep in mind the potential for poorer TST and QFT performance in the most vulnerable, namely the young and/or malnourished. The impact of age and malnutrition on TST and QFT, both tests of cellular immunity, is likely to be closely linked to the high proportion of discordant TST and QFT results in this population. Consistent with this, concordant and discordant TST and QFT results reflect differences in immune biomarker profiles. Reduced risk of TB seen in discordant positive adults, can for these reasons, not be assumed to apply to young children. For these reasons, the implementation of WHO's treatment recommendations should be intensified.[307]

Our work also links longitudinal QFT results with polyfunctional mycobacterial-specific CD4+ T cells in peripheral blood – a hitherto hot candidate when it comes to possible immune correlates of protection. Contrary to our starting hypothesis, our data do not support that the relative or absolute frequencies of PPD-specific polyfunctional CD4+ T-cells in peripheral blood can explain the reduced risk of TB progression observed in QFT reverters. Contrary, absolute frequencies of these cells correlated with the QFT-response, suggesting that this readout reflects the antigen load, similar to that shown for IGRA-responses in non-human primates.[188] Our data hereby supports earlier speculations on the requirement for persistent or recurrent MTB exposure to maintain specific IFNγ-responses.[30, 227, 264, 269]

6.1.2. Impact on new diagnostic and predictive tools in MTB infection and disease.

Through our work, we have for the first time confirmed a diagnostic potential for *RAB33A* when disease or infection with MTB is suspected in a pediatric setting. *RAB33A* seems to have a discriminatory potential across all relevant clinical TB categories both as a single biomarker and as part of biosignatures. We also suggest a

similar potential for *TGFB1*, but notably, this marker did not discriminate between MTB infection and disease, which is likely to be a requirement of a new diagnostic tool. Furthermore, it is of importance that the study was conducted on the Sub-Indian continent which is a relatively rare site in TB biomarker research. In order to be included as recommended diagnostic tools, potential biomarkers would need validation in multiple populations.[31]

A strong belief in a potential for polyfunctional mycobaterial-specific T cell responses in predicting TB progression or protection has inspired broad research based on resource intensive methods. Based on our finding that absolute frequencies of these cells correlated with the magnitude of the QFT response, we question the rationale for performing elaborate flow cytometry assays on peripheral blood samples in the search for "protective biomarkers". Notably, the extensive research aiming to identify immunologic biomarkers for diagnostic purposes mostly has a strong emphasis on cellular immunity. Better diagnostic accuracy is badly needed particularly in young children, but based on our work we suggest that age and malnutrition are likely to influence the performance of all cellular immunological markers. Future research on other approaches than host cell-mediated immune markers might prove more fruitful in this population.

6.2. Future research perspectives

Both epidemiological studies and advances in basic science are required to improve the tool-box of TB diagnostics. As stated by the Stop TB partnership, research efforts will continue to work towards a point-of-care test for active TB, explore biomarkers/bio-signatures which allow for accurate identification of patients at various stages of disease; from asymptomatic infection to disease, as well as biomarkers/bio-signatures capable of predicting protection or increased risk of TB progression in MTB infected or vaccinated subjects.[199]

In addition, findings included in this thesis point to other relevant research questions (listed below in unprioritized order):

- Further exploration and validation of immune biomarkers of MTB infection and disease with diagnostic potential in pediatric settings. Biomarkers suitable for point-of-care tests would be preferable.[308]
- Exploration of new diagnostic and predictive markers not dependent on cellular immunity. This will be particularly useful in young children, HIV patients and other immunocompromised individuals.
- Explore, in children <5 years, whether bacteriologically unconfirmed TB cases are different from smear/culture positive TB cases with regard to sociodemographic, clinical and nutritional characteristics.
- Explore host immune responses in smear/culture positive versus bacteriologically unconfirmed TB cases. Differences in disease manifestations are likely to be reflected in the host immune response or vice versa.
- What are the adequate Z-score cut-offs for the effect of malnutrition on the host immune system? Are the same cut-offs valid for all populations?
- The prognostic value of concordant and discordant TST and QFT results in children <3 years needs further investigation in order to optimize recommendations for management and treatment.
- Is preventive TB treatment required in otherwise healthy young children positive for TST and/or QFT in children without ongoing exposure? A close monitoring in a randomized study-setting can justify to with-hold treatment in these children.[309]
- Exploration of reduced cut-off for QFT test positivity in young children.
- Explore the immunological phenotype associated with indeterminate QFT results.
- Explore whether indeterminate QFT responses/low mitogen responses are reflected in more general immune biomarker profiles?
- Longitudinal studies of QFT reverters in high-endemic settings: Are they protected from re-infection? An answer to this question is important for the assessment of subjects at repeated risk of exposure: 1) Is repeated testing/examination indicated or not? 2) Should a booster vaccine be recommended?
- Longitudinal studies of QFT consistent positives in high-endemic settings to determine their risk of TB progression.

- Do the levels of polyfunctional mycobacterial-specific CD4+ T cells in the blood (or BAL) have the potential to predict TB progression (or MTB clearance) in MTB infected subjects?

In evaluating diagnostic tools for active TB, the study design should take into account that in real-life, subjects seek health because of symptoms, thus they are rarely healthy. The complex network of innate immunity more or less put into play in most disease states regardless of acute or chronic infectious, or non-infectious etiology, is likely to influence host immune biomarkers. Clifton E. Barry III recently reported that the pulmonary lesions in subjects with untreated XDR TB were almost completely rearranged within 2 months when assessed by high-resolution computed tomography (HRCT) and positron emission tomography (PET) (Clifton E. Barry III, unpublished data presented on the Keystone Symposia "Host Response in Tuberculosis", March 13-18, 2013, Whistler, BC, Canada). These gross changes were never reflected in IGRA responses (QFT and T-SPOT) questioning the ability of these tests and possibly specific T cells responses in blood, to reflect changes in extended disease. Notably, a very recent report suggests that sputum might be a more appropriate specimen for immune biomarker-based diagnostics.[306]

Regarding correlates of TB protection highly relevant in future TB vaccine trials, functional mycobaterial growth-inhibition assays using whole blood or PBMCs from study subjects might be both more promising and feasible than phenotyping of specific T cells.[268, 310]

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ERRATA

Please note the following corrections to the thesis:

Table of content.

Correction between point 3.3.1 and 3.3.2 *Clinical outcomes (diagnostic algorithm, Figure 9)* was wrongly assigned a headline. Likewise: *TB Antigen minus Nil (IU/mL) between point 3.8.2 and 3.8.3.*

1.1.5

page 15. The epidemiological numbers are updated according to the Global TB report 2014, which was not yet published when I submitted my thesis.

1.1.6.1.1

page 17. *antigen* changed to *antigens* and *PPD is an antigen* changed to *PPD contains antigens*

1.2.5.2

page 36. Added bolded text: *the onset of efficient adaptive immune responses* ***following infection*** *is crucial in controlling bacterial replication.*

1.3

page 36. Add bolded text: *Invasive procedures in the diagnosis of MTB infection* ***in absence of active TB*** *are hardly ethical.*

3.3.1

Figure 9, page 47. Added bolded text: *Clinical* ***entry*** *criteria.*

Figure 10, page 45. Add a conjunction line from the blue box "mixed outcomes" to the blue box to the left "clinical TB".

3.3.2

Table 5, page 49. Lay-out adjusted to increase the readability.

3.4

Table 6, page 47. Lay-out adjusted to increase the readability. In the row "When assessed", changed *Every 3 months* to *Every 2 (NCS) or 3 (ACS) months*. Added text in bold: **Failure to thrive **(defined in section 3.3.1, point 3.)**

3.8.5.5

Table 8, page 58. Lay-out adjusted to increase the readability.

3.8.5.6

page 59. Add bolded text: *lacked a valid positive control (****Valid positive control defined as*** *>1% cytokine-producing cells when stimulated with SEB).*

page 60: *is* changed to *was*

3.9

Figure 14, page 64. *Failure to thrive* in the lower left box should be in italic.

5.1.1

page 74. *More specifically, paper I-III cover the clinical setting of young children investigated for TB and subsequently shed light on current diagnostic tools (paper I, III and IV), potential future diagnostic (paper II) and predictive (paper IV) tools*. Changed to *Paper I, III and IV shed light on current diagnostic tools, whereas potential future diagnostic and predictive markers are explored in paper II and IV.*

page 75. *If the gradient of TST or IGRA results corresponds to the exposure,* changed to *If the magnitude of TST or IGRA results corresponds to the gradient of exposure,*

5.1.2.1

page 77. *dismissal* changed to *discharge.*

5.2

page 88. Final point changed to colon in *In paper III, the children are classified by their TST and/or QFT:*

page 92: added a parenthesis around the sentence: *For paper I, all the 746 children were eligible whereas 210 of the 746 were selected for the biomarker sub-studies in paper II and III (see section 3.3.1).* and deleted *(paper I-III)*

page 93: *crude mycobacterial antigen* changed to *crude mixture of mycobacterial antigens* and deleted bolded text *The TST which also applies the PPD* ***antigen***

page 94. *T cell* changed to *T cells*

page 98. Added bolded text *reduced sensitivity of the QFT* ***(compared to TST)***

page 101. Added bolded text *but* ***the receptors*** *can also be she* and made the font on *following the same kinetic pattern as* uniform: Times new roman 12.

page 102. Added bolded text *and* ***for young children,*** *our study suggests*

6.1.2

page 104. Deleted *in* an added bolded text in *Notably, in the extensive research aiming to identify immunologic biomarkers for diagnostic purposes* ***mostly*** *has a strong emphasis*

I

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Influence of Age and Nutritional Status on the Performance of the Tuberculin Skin Test and QuantiFERON®-TB Gold In-Tube in Young Children Evaluated for Tuberculosis in Southern India

Synne Jenum[1], MD, Sumithra Selvam[2], MSc, Diana Mahelai[3], Mphil, Nelson Jesuraj[3], MD, Vicky Cárdenas[4], PhD, JD, John Kenneth[3], MD, Anneke C. Hesseling[5], PhD, Timothy Mark Doherty[6], PhD, Mario Vaz[7], PhD, and Harleen MS. Grewal[8], MD, PhD, DTMH

[1]Center for Immune Regulation, Oslo University Hospital, Rikshospitalet-Radiumhospitalet Medical Center, Oslo, Norway.

[2]Division of Epidemiology, Biostatistics and Population Health, St. John's Research Institute, Bangalore, India.

[3]Division of Infectious Diseases, St. John's Research Institute, Bangalore, India.

[4]Areas, Rockville, USA.

[5]Department of Paediatrics and Child Health, Desmond Tutu TB Center, Stellenbosch University, Cape Town, South Africa.

[6]GlaxoSmithKline Pharma, Vaccines, Copenhagen, Denmark.

[7]Division of Health and Humanities, St. John's Research Institute, Bangalore, India.

[8]Department of Clinical Science, University of Bergen and Department of Microbiology, Haukeland University Hospital, Bergen, Norway.

Abbreviated title/running head title. Nutrition Affects TB Tests in Young Children

Corresponding Author. Harleen M.S Grewal MD, PhD, DTMH, Professor and Senior Consultant. Department of Clinical Science, Faculty of Medicine and Dentistry, University of Bergen, Norway and Department of Microbiology, Haukeland University Hospital, Bergen 5021, Norway.

Phone: +47 55-97-4631; Mobile: +47 99450554; Fax: +47 55-97-4689 ; E-mail: Harleen.Grewal@K2.uib.no

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Keywords. tuberculosis; tuberculin skin test; interferon gamma release assay; malnutrition, child; multivariate analyses.

ABSTRACT

Background. Reliable identification of *Mycobacterium tuberculosis* (MTB) infection or tuberculosis (TB) disease in young children is vital to assure adequate preventive and curative treatment. The tuberculin skin test (TST) and IFNγ-release assay (IGRA) may supplement the diagnosis of pediatric TB as cases typically are bacteriologically unconfirmed. However, it is unclear to what extent the performance of TST and QuantiFERON-TB Gold In-Tube (QFT) (Cellestis' IGRA test) depend on the demographic, clinical, and nutritional characteristics of children in whom they are tested.

Methods. During a 2-year prospective observational study of 4382 neonates in Southern India, children with suspected TB were investigated and classified by a standard TB diagnostic algorithm.

Results. Clinical TB was diagnosed in 13 of 705 children referred for case verification with suspected TB. TST and QFT had a susceptibility for clinical TB of 31% and 23%, respectively, in this group. Children <2 years were more likely to test QFT indeterminate. A height-for-age Z-score within the lowest quartile increased the odds ratio (OR) for a positive or indeterminate QFT result (OR 2.46 [1.19-5.06], OR 3.08 [1.10-8.58]), whereas the OR for a positive TST was reduced with a weight-for-height Z-score within the lowest quartile (OR 0.17 [0.06-0.47]).

Conclusion. The sensitivities of the TST and QFT for clinical TB in children <3 years of age were equally poor in this population. Stunted children were more susceptible to MTB infection, and more prone to indeterminate QFT results. TST was less reliable in children with wasting.

BACKGROUND

Children <2 years of age at the time of infection, typically develop tuberculosis (TB) disease due to primary infection, and not as a result of reactivation. Their risk of progression to TB and for developing severe disease is high. Therefore, reliable identification of *Mycobacterium tuberculosis* (MTB) infection or TB in young children is vital to assure adequate preventive and curative treatment. Pediatric TB often remains unconfirmed due to paucibacillary disease and technical difficulties in obtaining adequate specimens. Therefore, evidence of MTB infection by a positive tuberculin skin test (TST) or IFNγ-release assay (IGRA) may be used as a supplement in diagnosis(1). WHO recommends the use of TST over IGRAs in children in low- to middle- income countries as TST is cheaper and evidence suggests little difference in sensitivity(2). A trend towards lower sensitivity of all tests in children <5 years was highlighted in a recent meta-analysis, but the conclusion was limited by few included subjects(3). In addition, it is unclear to what extent the performance of TST and IGRAs depends on demographic, clinical and nutritional characteristics in young children.

In this study, we first evaluated the performance of TST and QuantiFERON-TB Gold In-Tube (QFT) in diagnosing clinical TB, in a cohort of children <3 years in a rural setting in Southern India. We then assessed the associations between TST and QFT results and sociodemographic, clinical, and nutritional (intra-uterine and post-natal) characteristics, in the same cohort.

METHODS

Study design and setting

This study was nested within the Neonatal Cohort Study (NCS), a population-based prospective study of 4,382 BCG-vaccinated neonates cluster randomized to active (2,215) or

passive (2,167) surveillance for 2 years, in the Palamaner Taluk, India (3.200^0N, 72.7500^0E, altitude 683m), from April 2007 to September 2010. Enrollment and consent procedures are previously described(4). The NCS covered a total population of ~400,000, with an estimated TB incidence of 136/100,000 (Andhra Pradesh, 2010)(5).

The present study is cross-sectional and included children enrolled in the NCS and evaluated for TB at the Case Verification Ward (CVW) at Emmaus Swiss Hospital, during the follow-up period. Referral criteria were either 1)exposure to a known TB case within the last year; 2)respiratory/infectious symptoms; 3)or failure to thrive (FTT) defined as any of the following; a)loss of weight or no weight gain for two consecutive visits; b)downward crossing of two percentile lines on the weight-for-age growth chart; or c)weight that tracked consistently below the 3rd percentile in the weight-for-age growth chart(6); or 4)a TST ≥10 mm at study close-out.

The study was approved by the institutional review board at St. John's Medical College, Bangalore, India, an independent ethics review committee, and the Ministry of Health Screening Committee, Government of India (No. 5/8/9/60/20006-ECD-1 dt.10.11.2006).

Diagnostic assessment

Clinical and anthropometric data were recorded. A chest radiograph (CXR), anterioposterior view, was interpreted by 3 independent radiologists. Agreement by 2 radiologists was required to classify a CXR as consistent with TB. A TST was performed by a trained nurse/doctor (2 TU/0.1mL tuberculin; Span Diagnostics Ltd, India) and read after 48 hours. Peripheral blood (3 ml) was drawn for the QuantiFERON®-TB Gold In-Tube (Cellestis, Australia) which was performed according to the manufacturer's instructions.

Gastric aspirates and induced sputa were collected on 2 consecutive days for fluorescent microscopy (Auramine) and culture on solid (Löwenstein-Jensen) and liquid (Mycobacterial Growth Indicator Tube, BD) medium. Positive results were confirmed by Ziehl-Neelsen staining and speciated using the HAIN kit (GenoType® MTBC, Ver1, Hain Life Sciences, Germany). Direct PCR (The COBAS® TaqMan® MTB Test, Roche 2007) was done on all culture-negative specimens in children with CXR consistent with TB. The HIV status was not assessed, but data from a house-hold contact study conducted in the same study area in the period 2010 to 2012, found a HIV prevalence <1% (TB Trials Study Group, unpublished data).

Clinical outcomes

A structured diagnostic algorithm including TST result (but not QFT), classified the children as having definite TB, probable TB or not TB. Clinical TB included definite TB (positive culture or PCR) and probable TB (CXR suggestive of TB and ≥1 of the following: known TB exposure, fever and/or cough ≥2 weeks, FTT and TST ≥10mm).

Statistical analysis

Low birthweight (<2500g) and being small for gestational age (SGA: Weight-for-gestational age <10^{th} percentile)(7) were used as indicators of intra-uterine nutritional status. The WHO child growth standard Z-scores were dichotomized using the cut-off <-2, which defines wasting (weight-for-height (WHZ)), stunting (height-for-age (HAZ)) and underweight (weight-for-age (WAZ) and BMI-for-age (BAZ))(6). Equal to the WHO definition, hemoglobin <10 g/100ml defined the cut-off for moderate-severe anemia(8). TST and QFT were handled as categorical variables; TST (binary), cut-off ≥10mm(9), QFT in 3 categories: positive (≥0.35 IU/mL), negative (<0.35 IU/mL) and indeterminate. Categorical data were

compared by Pearson's chi-square with Yates Continuity Correction or Fisher's exact test, where appropriate.

Kappa statistics were used in the concordance analyses of TST and QFT result and evaluated according to McGinn et al.(10).

Because malnutrition was left-shifted in the study-population, children within the lowest quartile were considered malnourished in new dichotomous variables which were included in the univariate and multivariate analyses (cut-offs: WHZ <-2.59, HAZ <-2.14, WAZ <-2.63 and BAZ <-2.51). Univariate associations between positive TST and/or QFT outcomes (indeterminate QFT results excluded) and surveillance arm (active or passive), gender, birthweight, SGA, age, socioeconomic factors (mothers/fathers education, housing, cooking fuel), known TB exposure, symptoms, nutritional status (WHZ, HAZ, WAZ, BAZ, anemia) and isolated NTMs, were assessed by logistic regression. Variables with significant univariate associations with the TST and/or QFT outcomes were considered for inclusion in the multivariate model if they could be assumed to have a casual impact on the outcomes. These assumed causal relationships were based on the literature and drawn by principles of a directed acyclic graph (DAG)(11). Notably, as the positive TST and/or QFT outcomes are imperfect read-outs of the clinical TB outcome (clinical TB disease, MTB infection or no infection/disease) for which there is no gold standard, we chose to illustrate the variables relationship both to the clinical TB outcome and the positive TST and/or QFT outcomes (Figure 1) (Causal relationships are indicated with arrows.) The variables' impact on clinical TB influence on the child's susceptibility for MTB infection and disease, whereas the variables impact on a positive TST and/or QFT result influence on the ability of the tests to correctly reflect the clinical TB outcome (test performance). Following the DAG principle, ancestor variables (variables that is a cause of another variable more closely linked to the outcome) and descendants of the outcome, were not included in the multivariate models(11).

These considerations left age, TB exposure, WHZ, HAZ and NTM presence in the multivariate model together with gender which is routinely included. WHZ and HAZ represent different entities of malnutrition, and important collinearity or interaction was not found when all possible 2-way interactions in each of the multivariate models were tested. Regarding the indeterminate QFT outcome, the assumed causal relationship is different (Figure 2). Being primarily the result of inadequate mitogen responses, this outcome is not specific for clinical TB outcome, but rather affected by all physiological conditions interfering with T-cell responses in general (Figure 2). Accordingly, known TB was not considered relevant whereas symptoms of any duration (categories defined in Table 6) were included because of previous reports(12). Missing data for each variable can be deduced from Table 1.

All p-values were calculated using two-tailed tests and a p-value of <0.05 was considered significant. Analyses were conducted using PASW Statistics version 18.0, 2009. SPSS Inc. Chicago, IL.

RESULTS

Characteristics of the study population

Of 746 children investigated at the CVW, 86 children (11.5%) had known TB exposure. Two or more referral criteria were present in 123 (16.5%). Of 623 children with a single referral criteria present, 452 (60.6%) had FTT, 80 (10.7%) had persistent cough and/or fever and 40 (5.4%) had known TB exposure. Among the 51 (6.8%) children admitted based on TST ≥10mm alone, 44 (86.3%) were referred at study closure. Of the 746 children, 41 where excluded from further analyses as missing data prevented classification by the diagnostic algorithm (40), or loss to follow-up (1) (Figure 3). None of these children had a CXR suggestive of TB, and only one child had known TB exposure. Subsequently, 705 children

were included in further analyses. Their clinical characteristics are provided in Table 1. Except for CXR changes, TST and QFT test results, there were no significant differences in sociodemographic, clinical or nutritional variables between children with or without clinical TB. The mean age was 14.8 months, about 50% of the mothers and fathers had primary or secondary school, 76.6% lived in brick houses but only 7% of the families used gas to cook. Known TB exposure was present in 12.1%. A striking 76.2% had FTT. Poor nutritional status was also reflected in the WHO growth-chart Z-scores (40-50% had wasting/underweight and 30% had stunting) and in the proportion of children with moderate-severe anemia (32.8%).

Performance of TST and QFT in clinical TB cases

Thirteen clinical TB cases, (4 definite and 9 probable) were identified; 4 were TST positive and 3 QFT positive. The sensitivity of TST and QFT for clinical TB was 31% and 23%, respectively (Table 2). Among children with definite TB, 3 had negative smears but positive cultures, and were TST and QFT positive. The fourth child with definite TB was TST and QFT negative (sensitivity for definite TB: 75%). This 18 months old boy was the only child in our study with a positive smear (gastric aspirate grade 3+), illustrating the shortcomings of TST and QFT also in definite TB cases despite an adequate T-cell response to mitogen (>10). He had been initiated on anti-TB treatment 2 weeks prior to admission which likely explains the negative culture, but MTB was confirmed by PCR. Of 9 children classified as probable TB, one infant was TST positive, 7 were QFT negative and 2 QFT indeterminate (Table 2).

Overall TST and QFT results

In the 705 children, 69 of 702 (9.8%) were TST positive. QFT was positive in 36 and indeterminate in 22 of 691 (5.1% and 3.1%, respectively). Of the children with an indeterminate QFT, 19 (86.4%) were aged <12 months and the remaining 3 (13.6%) were

aged 12-23 months. The difference in the distribution of indeterminate results within the 3 age categories were thus highly significant (Fisher's Exact test, $p<0.0005$). Low mitogen response was the predominant cause of an indeterminate QFT result in 21 children (95.5%). Notably, indeterminate QFT was more frequent in children with clinical TB (15%) compared to children without (3%).

TST and QFT agreement

Valid results for both TST and QFT were obtained in 666 children. The 39 children without corresponding TST and QFT results were thus omitted from this analysis. Agreement between the tests was only fair (κ=0.30, 95%CI 0.18-0.42), indicating that exchanging one test for the other is unlikely to provide the same information. When restricted to those with known TB exposure, which increases the pretest probability of the tests, the agreement was substantial (κ=0.75, 95%CI 0.42-0.95) (Table 3). This highlights the importance of focusing on TB exposure rather than immunological tests in young pediatric populations.

Determinants for a positive TST and/or QFT

Unadjusted odds ratio estimates for the variables: surveillance arm (active or passive), gender, birthweight, SGA, age, socioeconomic factors (mothers/fathers education, housing, cooking fuel), known TB exposure, symptoms, nutritional status (WHZ, HAZ, WAZ, BAZ, anemia) and isolated NTMs, were performed by logistic regression. If significant, these estimates, with 95% confidence intervals, are presented in Table 5 and 6 together with unadjusted and adjusted analyses for all variables included in the final multivariate model: gender, age (categorical), known TB exposure, nutritional status (WHZ, HAZ) and NTM isolated from culture. Data not shown are available on request.

Adjusted for the other factors, boys had increased OR for a positive TST. Younger age associated negatively with a positive TST and/or QFT, more prominently with TST than QFT, both in children <12 and 12-23 months compared to children ≥24 months. TB exposure was a determinant for a positive TST and/or QFT. Within the study population, having a WHZ<-2.59 reduced the OR for a positive TST whereas having a HAZ<-2.14 increased the OR for a positive QFT and positivity for both tests, but not for TST alone. Birthweight <2500g and SGA were included in the models one at a time, but no significant associations was present and the other coefficients remained unchanged (data not shown). All possible 2-way interactions in the models were tested, but only a slight interaction between WHZ and HAZ (p=0.048) for a positive QFT was found. The model including the interaction gave a better fit (Likelihood Ratio test Statistics: X^2=5.34, p<0.025), but did not change the coefficients. Stratified analyses indicated that the effect of having a HAZ<-2.14 on a positive QFT, was not evident in children with WHZ<-2.59 (OR 0.55, 95%CI 0.06-5.13 versus OR 4.0, 95%CI 1.71-9.35).

Determinants for an indeterminate QFT

Results from unadjusted and adjusted analyses are given in Table 7.

The multivariate model included gender, age (continuous), fever and/or cough of any duration, nutritional status (WHZ and HAZ) and NTM isolated from culture. Adjusted for the other factors, the OR of an indeterminate result was reduced by 0.83 with every month of age (95%CI 0.76-0.90). Fever and cough as well as a HAZ within the lowest quartile increased the OR for an indeterminate QFT. Neither birthweight <2500g nor SGA showed any association or changed the other coefficients, and were therefore omitted in the final model.

The impact of alternative nutritional variables on TST and QFT outcomes

WAZ and BAZ are alternative markers of nutritional status and indicate underweight if the Z-score is <-2)(6). Exchanging WHZ and HAZ for WAZ or BAZ in the multivariate models, WAZ (cut-off<-2.63) and BAZ (cut-off<-2.51) acted similarly by reducing the OR for a positive TST (OR_{WAZ} 4.00, 95%CI 1.61-9.95, OR_{BAZ} 3.02, 95%CI 1.23-7.44) and increased the OR for an indeterminate QFT result (OR_{WAZ} 0.35, 95%CI 0.16-0.77, OR_{BAZ} 0.20, 95%CI 0.07-0.54) in children with Z-scores below the cut-off.

DISCUSSION

To our knowledge, this is the first extensive study of TST and QFT performance in young Indian children. The sensitivities of TST and QFT test for clinical TB were equally poor, which may be attributable to the influence of younger age and malnutrition. QFT results did not add significantly to the information already provided by TST, supporting current WHO guidelines in high-burden TB settings(2). We report for the first time that a low HAZ score, which indicates stunting, a result of chronic malnutrition, is a determinant for an indeterminate QFT result when corrected for other factors.

The sensitivities of TST and QFT for clinical TB were 31% and 23%, which is comparable to studies conducted in young African children(13-16). The diagnostic criteria in this study are slightly less stringent than recently recommended by an expert panel(17), but the proportion of confirmed TB cases (30%) is similar to other studies of pediatric TB(18). The lack of a gold standard for MTB infection and the diagnosis of smear and culture negative TB complicates the evaluation of TST and QFT performance.

Determining the clinical relevance of bacteriologically unconfirmed TB is an important issue in pediatric TB. Because pediatric TB is often paucibacillary(1), it can be argued that false negative QFT results could be expected as increasing evidence supports a positive association between the antigen load and the magnitude of MTB-specific IFNγ-

responses(19;20). Accordingly, children with sputum-negative TB could simply be in the early stages of TB. Given that cases in this study were identified by active case finding, this is very likely(1). While published studies indicate an increased risk of TB disease in subjects with TST indurations ≥12mm(21), or high IFNγ-responses following recent infection(19;20) these studies were conducted almost entirely in adults. Interestingly, adults with discordant TST and IGRA results had the lowest rates of TB in a household contact study in the Gambia(20). From this perspective one could argue that there is an over-diagnosis of TB in young children. However, epidemiological estimates suggest the opposite(1) and TST and/or QFT negativity is not restricted to unconfirmed TB cases(13;15;16;22), as shown in this study. Furthermore, we report increased OR for indeterminate QFT in children with intercurrent disease (fever and/or cough of any duration). This questions the validity of extrapolation of immune responses in asymptomatic, latently infected adults, to immune responses in sick children. Moreover, unlike adults, children <2 years at time of infection who progress to TB, can almost universally be assumed to do so after a primary infection(1). The nature of a primary and/or immature immune response(23), could cause MTB-specific IFNγ-producing T-cells to be present in lower numbers in young children.

Ideally, determinants for TST and QFT outcomes should be evaluated in clinical TB cases, but this is possible only in prospective community-based settings where the TB incidence is high. Therefore, we evaluated associations between determinants and TST/QFT results regardless of TB disease status. As expected, known TB exposure associated with a positive TST and/or QFT when corrected for other factors. Our study indicates that there might be factors which mask the true relationship between known TB exposure and a positive TST since this association was evident only in adjusted analysis.

Boys were more likely to be TST positive than girls when corrected for other factors. Basu *et al.*, recently reported that boys were more likely to be QFT positive, but found no

such association with TST(24). There are well-known gender differences in TB epidemiology, but it is unclear to which extent these are attributable to genetic as opposed to socioeconomic and cultural factors(25).

Children <2 years were less likely to be TST and/or QFT positive and more likely to have an indeterminate QFT result than children aged 2-3 years. This is not surprising as the immune responses in infancy are considered immature and evolving(23). Previous findings in young children are contradictory with regard to a positive association between age and a positive TST(14;24). Regarding QFT, some studies reported no association with age, but few young children were included(16;26). In a large study of children <5 years, age associated positively with a positive TST and QFT after adjusting for BCG vaccination and gender(24). Many studies have suggested indeterminate QFT to be more frequent in young individuals (16;22;24).

The burden of malnutrition in this study was considerable. Malnourished children are more susceptible to infections because of altered immune responses(27), and would thus be expected to have increased susceptibility to MTB infection and subsequently an increased rate of positive TST and/or QFT. On the other hand, blunted immune responses by malnutrition are likely to cause suboptimal performance of immunological tests as reported for immunocompromised children(28). We report that HAZ <-2.16 increased the OR for a positive TST and/or QFT, when corrected for other factors. A HAZ <-2 is a marker of stunting, the result of chronic malnutrition. Our findings therefore confirm the increased susceptibility to MTB infection in children with chronic malnutrition. At the same time, these children were more prone to indeterminate QFT (overwhelmingly due to lower mitogen-driven responses), which together with a substantially reduced OR for a positive TST when WHZ was <-2.61, is suggestive of a blunted immune response in these children. The clinical consequences of blunted immunity are demonstrated by a higher mortality in hospitalized

children with indeterminate QFT, regardless of the underlying disease(16). Studies on the effect of malnutrition on TST are conflicting(29;30), but reduced sensitivity of TST for clinical TB in children with WAZ <-2 have been reported(13). Few studies (all evaluating WAZ <-2 without considering other Z-scores) have evaluated malnutrition as a determinant for QFT results(16;26). The definitions of malnutrition in studies vary considerably, and conflicting results might be attributed to different effects of chronic and sub-acute malnutrition. Furthermore, the standard Z-score cut-off <-2 has not been evaluated with regard to immunological changes related to malnutrition. We have demonstrated that the effect of malnutrition depends on the variable used. When WAZ or BAZ replaced WHZ and HAZ in our adjusted models, the effect of chronic malnutrition on a positive QFT result was lost.

Children with at least one NTM isolated from culture (23.5%), but had no increased OR for being TST positive when corrected for other factors, suggesting that reduced specificity of TST by NTM cross-reactivity plays little role in this setting. Similarly, no apparent impact of NTM on TST was reported in Gambian children(31). QFT (positive/indeterminate) was not influenced by the presence of NTM, and all children with RD1-positive NTM (*M. kansasii*) were QFT negative. No child fulfilled the ATS/IDSA criteria(32) for NTM disease, suggesting that NTMs were commensals in this setting.

The strength of this study is the community-based prospective design with a considerable number of enrolled children in a country with high TB burden but low HIV prevalence (<1%) (TB Trials Study Group, unpublished data). This "real-life" context makes our findings very clinically-relevant. The high prevalence of malnutrition and colonization by NTMs may limit the generalizability to other settings, but these conditions are typical for many areas with high-to-moderate TB burden. The prevalence of helminth infection is presumably high(33) and might interact with host immune responses(34;35), but we were

unable to collect stool samples in this cohort. Well-quantified TB exposure is a reliable surrogate measure of MTB infection(36), as reflected in this study by the increased kappa agreement between TST and QFT when the analysis was restricted to TB exposed children. Unfortunately graded data on TB exposure was not available in this study. TST ≥10mm outcome was both a referral criteria and 1 of 4 diagnostic criteria (≥1 criteria in addition to characteristic CXR changes) required for a diagnosis of clinical TB. The TST estimates could therefore be subject to selection bias, which is a limitation in our study. This compromise in design was required in order to avoid losing TB cases; the primary aim of the NCS was to establish the true incidence of TB in this cohort as part of preparing the site for future vaccine trials. This selection bias could result in an overestimation of the TST's sensitivity for clinical TB, but as none of the probable TB cases depended on a positive TST for their diagnosis, this does not affect the TST sensitivity estimate. Furthermore, compared to the OR estimates of the associations between a positive TST and the assessed variables, the OR estimates for a positive QFT outcome may be overestimated because TST-QFT+ discordant positives children within the NCS were only referred if they had known TB exposure or were symptomatic, whereas TST+QFT- children might have had no other referral criteria. Notably, referral based on a positive TST only was present in 51 of 746 children, which limits this effect.

CONCLUSIONS

This study highlights that in assessing children with suspected TB, it is vital to keep in mind the potential for poorer TST and QFT performance in the most vulnerable, namely the young and/or malnourished. Accordingly, the implementation of WHO's treatment recommendations should be intensified(37). Withholding treatment in exposed and/or TST/IGRA positive but otherwise healthy children can only be justified if careful clinical

follow-up is possible. TST and QFT are immunological tests of cellular immunity which provide indirect evidence of MTB infection. Currently, extensive research is aimed at identifying immunologic biomarkers for diagnostic purposes, with a strong emphasis on cellular immunity. Better diagnostic accuracy in this population is badly needed, but based on the results of this study, we suggest that age and malnutrition are likely to influence the performance of all cellular immunological markers. Future research on other approaches than host cell-mediated immune markers might prove more fruitful in this population.

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Contributors. *TB Trials Study Group:* Doherty TM (GlaxoSmithKline, Copenhagen, Denmark) Grewal HMS (Department of Clinical Science, Faculty of Medicine and Dentistry, University of Bergen, and Department of Microbiology, Haukeland University Hospital, Bergen, Norway), Lindtjørn B, (University of Bergen, Norway), Hesseling AC (Department of Paediatrics and Child Health, Stellenbosch University, Cape Town, South Africa), Jacob A (Emmaus Swiss Leprosy Project, Palamaner, India), Jahnsen F (Center for Immune Regulation, Oslo University Hospital, Rikshospitalet-Radiumhospitalet Medical Center, Oslo,

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FIGURE LEGENDS

Figure 1.

A Directed Acyclic Graph (DAG) illustrating the causal relationships between the determinants for the outcomes clinical TB and positive TST and/or QFT. A causal relationship between a determinate (variable names listed in parenthesis) and an outcome is indicated with arrows. Ancestors (variables that is a cause of another variable more closely linked to the outcome) and descendants of the outcome not included in the multivariate models, are in italic. Variables significantly associated to the outcomes in univariate analysis are in bold. Dashed arrows indicate more uncertain relationships.

Figure 2.

A Directed Acyclic Graph (DAG) illustrating the causal relationships between the determinants for the outcome indeterminate QFT. A causal relationship between a determinate (variable names listed in parenthesis) and an outcome is indicated with arrows. Ancestors (variables that is a cause of another variable more closely linked to the outcome) and descendants of the outcome not included in the multivariate models, are in italic. Variables significantly associated to the outcomes in univariate analysis are in bold. Dashed arrows indicate more uncertain relationships.

Figure 3.

Flow-chart for the 746 study participants. The diagnostic procedures applied at the *Case Verification Ward (CVW) are listed.

Table 1. Characteristics of children referred to the Case Verification Ward (CVW) classified according to the diagnostic TB algorithm (n=705).[a]

	Total n=705 (%)	Clinical TB[1] n=13 (%)	No TB n=692 (%)	p value
Surveillance arm (N=705)				
Active	609 (86.4)	10 (76.9)	599 (86.6)	0.40
No of visits to the CVW (N=705)				
1 visit	663 (94.0)	10 (76.9)	653 (94.4)	0.07
2 visits	40 (5.7)	3 (23.1)	37 (5.3)	
3 visits	2 (0.3)	-	2 (0.3)	
Sex (N=705)				
Male	374 (53.0)	7 (53.8)	367 (53.0)	1.00
Birthweight (g) (N=703)				
Mean	2807	2735	2809	0.13
Min-Max	1500-4500	(1500-4000)	(2000-4500)	
Small for gestational age	72 (10.2)	3 (23.1)	69 (10.0)	0.14
Age (months) (N=705)				
Mean	14.8	16.8	14.8	0.84
Min-Max	(1-35)	(8-28)	(1-35)	
0-11 months	220 (31.2)	4 (30.8)	216 (31.2)	0.53
12-23 months	392 (55.6)	6 (46.2)	386 (55.8)	
≥ 24 months	93 (13.2)	3 (23.1)	90 (13.0)	
Mothers education (N=705)				
High school or higher	143 (20.3)	1 (7.7)	142 (20.5)	0.37
Primary, secondary	351 (49.8)	9 (69.2)	342 (49.4)	
Illiterate	211 (29.9)	3 (23.1)	208 (30.1)	
Fathers education (N=701)				
High school or higher	195 (27.7)	1 (7.7)	194 (28.0)	0.18
Primary, secondary	372 (52.8)	8 (61.5)	364 (52.6)	
Illiterate	134 (19.0)	4 (30.8)	130 (18.8)	
Housing (wall) (N=705)				
Bricks	540 (76.6)	11 (84.6)	529 (76.4)	0.74
Other	165 (23.4)	2 (15.4)	163 (23.6)	
Cooking fuel (N=705)				
Electricity(none) or gas	49 (7.0)	-	49 (7.1)	1.00
Other	656 (93.0)	13 (100.0)	643 (92.9)	
Known contact with TB case (N=705)				
Yes	85 (12.1)	2 (15.4)	83 (12.0)	0.66
Symptoms (fever and/or cough) (N=705)				
2 of 2 symptoms	145 (20.6)	3 (23.1)	142 (20.5)	1.00
1 of 2 symptoms	142 (20.1)	2 (15.4)	140 (20.2)	
None	418 (59.3)	8 (61.5)	410 (59.2)	
Fever and/or cough ≥ 2weeks (N=705)				
Yes	175 (24.8)	4 (30.8)	171 (24.7)	0.75
Failure to thrive (FTT) (N=705)				
Yes	537 (76.2)	8 (61.5)	529 (76.4)	0.20
Chest X-ray (AP) (N=704)				
Consistent with TB	11 (1.6)	11 (84.6)	-	**<0.0005**
Abnormal, not TB	12 (1.7)	-	12 (1.7)	

Normal	681 (96.6)	2 (15.4)	679 (98.1)	
Non-tuberculous mycobacteria (NTM) (N=705)				
≥1 positive specimen	166 (23.5)	2 (15.4)	164 (23.7)	0.74
Weight-for-height Z score (WHZ) (N=703)				
Mean	-1.80	-1.86	-1.80	0.38
Min-Max	-4.93-3.61	(-4.06-1.59)	(-4.93-3.61)	
< -2 (wasting)	305 (43.3)	7 (53.8)	298 (43.1)	0.57
Height-for-age Z score (HAZ) (N=703)				
Mean	-1.25	-2.23	-1.24	0.81
Min-Max	-6.32-3.13	(-2.95-0.35)	(-6.32-3.13)	
< -2 (stunting)	197 (27.9)	6 (46.2)	191 (27.6)	0.21
Weight-for-age Z score (WAZ) (N=705)				
Mean	-1.95	-2.11	-1.95	0.47
Min-Max	-5.00-1.67	(-3.99-1.33)	(-5.00-1.67)	
< -2 (underweight)	354 (50.2)	8 (61.5)	346 (50.0)	0.58
BMI-for-age Z score (BAZ) (N=703)				
Mean	-1.70	-1.70	-1.70	0.44
Min-Max	-4.95-4.63	-3.86-1.55	-4.95-4.63	
< -2 (underweight)	284 (40.3)	7 (53.8)	277 (40.0)	0.40
Hgb (g/100ml) (N=601)				
Mean	10.7	10.0	10.7	0.16
Min-Max	4.0-18.9	8.0-11.6	4.0-18.9	
<10 g/100ml (moderate-severe anemia)	197 (32.8)	6 (54.5)	191 (32.4)	0.11
Tuberculosis Skin Test (TST) (N=702)				
Median	4.0	7.5	4.0	0.07
Positive (cut-off ≥10mm)	69 (9.8)	4 (30.8)	65 (9.4)	**0.031**
QuantiFERON Gold In-Tube (N=691)				
Positive (≥ 0.35IU/mL)	36 (5.1)	3 (23.1)	33 (4.8)	**0.003**
Negative (< 0.35IU/mL)	633 (89.8)	8 (61.5)	625 (90.3)	
Indeterminate	22 (3.1)	2 (15.4)	20 (2.9)	
Mitogen minus Nil (median)	10	10	10	0.40
TB-antigen minus Nil (median)	0.01	0.02	0.01	0.29

[a]Comparison of distribution between infants with and without clinical TB disease perforemd by T-test, Mann-Whitney U test or Fisher's Exact test where appropriate. 2-sided p-values <0.05 are shown in bold. [1]No missing data among infants with clinical TB disease.

Table 2. Characteristics of clinical TB cases

	Surveillance	Gender	Age (months)	data from visit	TST (mm)	Quantiferon-TB Gold In-Tube				Chest X ray result	Known TB exposure	Fever/cough ≥2 weeks	FTT[1]	WHZ[2]	HAZ[3]	WAZ[o]	SGA^	Hgb (g/dl)	smear*	culture
						Test result	Nil	Test value (IU/mL)	Mitogen minus Nil (IU/mL)											
Definite TB	Passive	M	18	2nd	9	-	0.15	0.11	> 10	Abnormal, TB	+	+	+	-2,35	-1,21	-2,26	-	11.3	+++ (confirmed by PCR)	-
	Active	M	9	1st	10	+	0.32	8.41	> 10	Normal	-	+	+	-2,93	-0,83	-2,58	-	11.3	-	+ (3/4)
	Active	M	28	2nd	13	+	0.25	> 10	> 10	Normal	-	-	-	-1,94	-2,7	-2,84	-	9.4	-	+ (3/4)
	Passive	M	26	1st	20	+	0.4	> 10	> 10	Abnormal, TB	-	-	+	0,36	-2,61	-1,14	-	not done	-	+ (2/4)
Probable TB	Active	F	18	1st	0	-	0.08	-0.01	> 10	Abnormal, TB	-	+	+	-2,74	-2,04	-2,99	+	9.5	-	-
	Active	F	10	1st	2	-	0.13	0.02	1.18	Abnormal, TB	-	+	+	-2,16	-1,65	-2,49	+	8.0	-	-
	Active	M	14	2nd	4	-	0.96	-0.6	> 10	Abnormal, TB	+	+	-	-4,06	-2,44	-3,99	-	11.1	-	-
	Active	F	16	1st	4	-	0.06	-0.02	3.92	Abnormal, TB	-	+	-	-2,65	0,18	-1,81	-	11.6	-	-
	Active	M	15	1st	4	-	0.18	0.09	> 10	Abnormal, TB	-	+	+	-2,99	-2,36	-3,23	-	9.1	-	-
	Active	F	13	1st	5	-	0.08	0.01	> 10	Abnormal, TB	-	-	+	-1,46	-1,72	-1,91	+	10.2	-	-
	Passive	F	24	1st	16	-	0.15	0.23	> 10	Abnormal, TB	-	+	-	-1,26	-2,95	-2,54	-	9.5	-	-
	Active	M	8	1st	5	indet[a]	0.29	-0.12	0.02	Abnormal, TB	-	+	-	-1,57	0,35	-1,03	-	8.8	-	-
	Active	F	10	1st	6	indet[a]	0.1	-0.01	0.49	Abnormal, TB	-	+	-	1,59	0,34	1,33	-	not done	-	PCR indet[a]

[1] FTT: failure to thrive; [2] WHZ: weight-for-height Z-score; [3]HAZ: height-for-age Z-score; [o]WAZ: weight-for-age Z-score; ^SGA: small for gestational age (<10th percentile).*From gastric aspirate and/or induced sputum; [a]indeterminate.

Table 3. The performance of the Tuberculin skin test (TST) and the QuantiFERON TB Gold In-Tube (QFT) in the diagnosis of clinical TB disease.

	TB status		Sensitivity	Specificity	PPV	NPV	κ (95%CI)*
	Clinical TB N	No TB N					
Tuberculin Skin Test							
≥10mm	4	65	30.8%	90.6%	5.8%	98.6%	**0,07**
<10mm	9	624	(4/13)	(624/689)	(4/69)	(624/633)	**(-0.02, 0.16)**
QuantiFERON TB Gold In-Tube							
≥0.35IU/mL	3	33	23.1%	92.2%	8.3%	98.7%	**0,11**
<0.35IU/mL	8	625	(3/13)	(625/678)	(3/36)	(625/633)	**(-0.02, 0.27)**
Indeterminate	2	20					

*Indeterminate QFT results are excluded from analysis of kappa agreement. Confidence intervals that do not overlap the null value κ=1 are shown in bold.

Table 4. Kappa agreement (κ)* between the QuantiFERON TB Gold In-Tube and the Tuberculin skin test (TST) in A: all children tested with both tests; B: children with known TB exposure.

	A: Tuberculin skin test, all (N=688)		B: Tuberculin skin test, children with known TB exposure (N=79)	
	≥10mm, N (%)	<10mm, N (%)	≥10mm, N (%)	<10mm, N (%)
QuantiFERON TB Gold In-Tube				
≥0.35IU/mL	18 (26.9)	18 (2.9)	7 (63.6)	1 (1.5)
<0.35IU/mL	48 (71.6)	582 (93.7)	3 (27.3)	65 (95.6)
Indeterminate	1 (1.5)	21 (3.4)	1 (9.1)	2 (2.9)
κ (95%CI)	**0.30 (0.18, 0.42)**		**0.75 (0.42, 0.95)**	

*Indeterminate QFT results are excluded from analysis of kappa agreement. Confidence intervals that do not overlap the null value κ=1 are shown in bold.

Table 5. Unadjusted odds ratio estimates and 95% confidence intervals for significant associations between a positive Tuberculosis Skin Test (TST) or QuantiFERON TB Gold In-Tube (QFT) as dependant variables and sociodemographic, clinical, nutritional and mycobateriological factors (not *M. Tuberculosis*)[a]. Adjusted odds ratio estimates and 95% confidence intervals are shown for all variables included in the multivariate model. Odds ratio estimates and 95% confidence intervals for variables not included in the table are available on request.

	TST ≥10mm vs. TST<10mm (N=702)		QFT ≥0.35 IU/mL vs. QFT <0.35 IU/mL (N=669)	
	Unadjusted OR (95%CI)	Adjusted OR (95%CI)	Unadjusted OR (95%CI)	Adjusted OR (95%CI)
Surveillance				
Active	**0.12 (0.07, 0.21)**		**0.33 (0.16, 0.70)**	
Gender				
Male	1.54 (0.92, 2.56)	**1.91 (1.03, 3.53)**	1.47 (0.74, 2.93)	1.55 (0.76, 3.17)
Age categories				
0-11 months	**0.05 (0.02, 0.10)**	**0.04 (0.02, 0.08)**	**0.31 (0.13, 0.75)**	**0.34 (0.13, 0.86)**
12-23 months	**0.05 (0.03, 0.09)**	**0.05 (0.02, 0.09)**	**0.23 (0.11, 0.51)**	**0.25 (0.11, 0.56)**
≥ 24 months				
Fathers education				
High school or higher	2.06 (0.84, 5.01)		0.83 (0.32, 2.17)	
Primary, secondary	**2.31 (1.01, 5.27)**		0.79 (0.34, 1.8)	
Illiterate[1]				
Known contact with TB case				
Yes	1.62 (0.83, 3.15)	**2.60 (1.14, 5.93)**	**2.37 (1.04, 5.42)**	**3.03 (1.26, 7.28)**
Failure to thrive (FTT)				
Yes	**0.16 (0.10, 0.27)**		**0.48 (0.24, 0.98)**	
Weight-for-height Z score (WHZ)				
<-2.59 (n=173)	**0.22 (0.09, 0.55)**	**0.17 (0.06, 0.47)**	0.90 (0.40, 2.02)	0.94 (0.41, 2.15)
Height-for-age Z score (HAZ)				
<-2.14 (n=176)	**1.71 (1.01, 2.90)**	1.42 (0.74, 2.71)	**2.57 (1.30, 5.08)**	**2.46 (1.19, 5.06)**
BMI-for-age Z score (BAZ)				
<-2.51 (n=175)	**0.21 (0.09, 0.54)**		0.75 (0.32, 1.76)	
Non-tuberculous mycobacteria (NTM)				
≥1 positive specimen	**0.46 (0.22, 0.95)**	0.46 (0.20, 1.04)	1.05 (0.48, 2.27)	1.12 (0.78, 1.12)

[a]Binary logistic regression was applied. Confidence intervals that do not overlap the null value OR=1 are shown in bold.
[1]Reference group. [2]Moderate-severe anemia.

Table 6. Unadjusted and adjusted odds ratio estimates and 95% confidence intervals for the significant associations between a positive Tuberculosis Skin Test (TST) and QuantiFERON TB Gold In-Tube (QFT) as dependant variable and sociodemographic, clinical, nutritional and mycobateriological factors (not M. Tuberculosis). Adjusted odds ratio estimates and 95% confidence intervals are shown for all variables included in the multivariate model. Odds ratio estimates and 95% confidence intervals for variables not included in the table are available on request[a].

	TST ≥10mm and QFT ≥0.35 IU/mL vs. other combinations of TST and QFT test results (N=666)	
	Unadjusted OR (95%CI)	Adjusted OR (95%CI)
Surveillance		
Active	**0.14 (0.05, 0.37)**	
Gender		
Male	1.16 (0.45, 2.98)	1.42 (0.51, **3.95**)
Age categories		
0-11 months	**0.12 (0.03, 0.42)**	**0.10 (0.02, 0.34)**
12-23 months	**0.08 (0.02, 0.24)**	**0.07 (0.02, 0.25)**
≥ 24 months		
Known contact with TB case		
Yes	**5.34 (2.00, 14.23)**	**9.41 (2.93, 30.2)**
Failure to thrive (FTT)		
Yes	**0.17 (0.07, 0.45)**	
Weight-for-height Z score (WHZ)		
<-2.59 (n=173)	2.19 (**0.92**, 5.23)	2.48 (0.98, 6.28)
Height-for-age Z score (HAZ)		
<-2.14 (n=176)	**3.17 (1.24, 8.12)**	**3.46 (1.19, 10.0)**
Non-tuberculous mycobacteria (NTM)		
≥1 positive specimen	0.70 (0.23, 2.09)	0.69 (0.21, 2.24)

[a]Binary logistic regression was applied. Confidence intervals that do not overlap the null value OR=1 are shown in bold. [1]Reference group. [2]Moderate-severe anemia.

Table 7. Unadjusted and adjusted odds ratio estimates and 95% confidence intervals for the significant associations between an indeterminate QuantiFERON TB Gold In-tube assay (QFT) as dependant variables and demographic, clinical, nutritional and mycobateriological factors. Adjusted odds ratio estimates and 95% confidence intervals are shown for all variables included in the multivariate model. Odds ratio estimates and 95% confidence intervals for variables not included in the table are available on request.

	Indeterminate QFT vs. valid QFT (N=691)	
	Unadjusted OR (95%CI)	Adjusted OR (95%CI)
Surveillance		
Active	3.35 (0.45, 25.2)	
Gender		
Male	1.61 (0.66, 3.88)	1.41 (0.56, 3.58)
Small for gestational age		
<10th percentile (WHO)	2.02 (0.67, 6.16)	
Age (months)		
	0.84 (0.77, 0.91)	**0.83 (0.76, 0.90)**
Symptoms (fever and/or cough)		
2 of 2 symptoms	**3.48 (1.32, 9.20)**	**3.20 (1.14, 8.95)**
1 of 2 symptoms	1.90 (0.61, 5.92)	1.34 (0.40, 4.50)
None[1]		
Fever and/or cough ≥ 2weeks		
Yes	**2.70 (1.14, 6.36)**	
Failure to thrive (FTT)		
Yes	**0.34 (0.15, 0.81)**	
Weight-for-height Z score (WHZ)		
<-2.59 (n=173)	2.19 (0.92, 5.23)	2.48 (0.98, 6.28)
Height-for-age Z score (HAZ)		
<-2.14 (n=176)	1.41 (0.57, 3.51)	**3.08 (1.10, 8.58)**
Weight-for-age Z score (WAZ)		
<-2.63 (n=179)	**3.03 (1.29, 7.12)**	
BMI-for-age Z score (BAZ)		
<-2.51 (n=175)	**3.17 (1.35, 7.45)**	
Non-tuberculous mycobacteria (NTM)		
≥1 positive specimen	0.70 (0.23, 2.09)	0.69 (0.21, 2.24)

[a]Binary logistic regression was applied. Confidence intervals that do not overlap the null value OR=1 are shown in bold. [1]Reference group. [2]Moderate-severe anemia.

Figure 1.

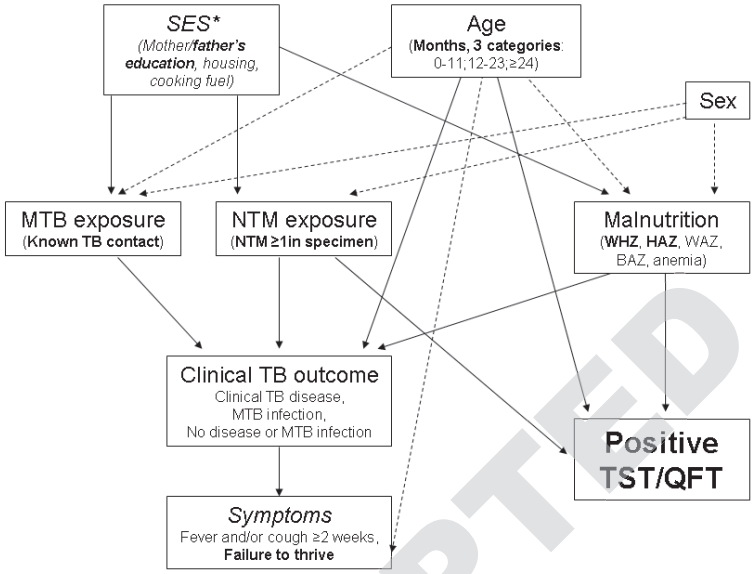

Figure 2.

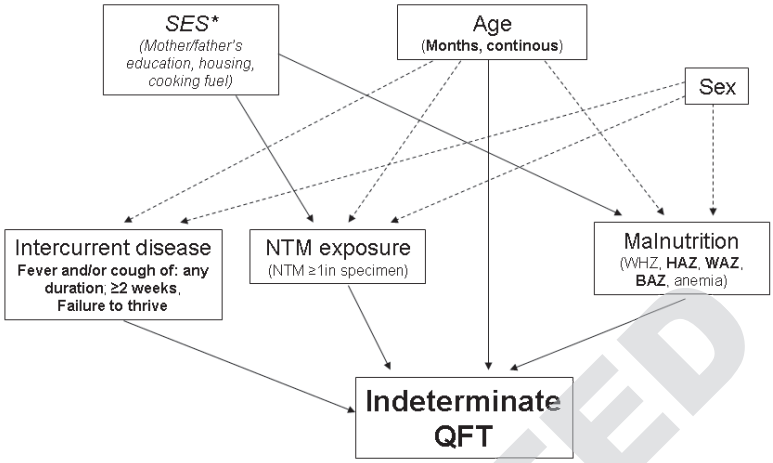

Figure 3.